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TRANSMITTAL FORM

Application Serial Number	10/067,527
Filing Date	February 4, 2004
First Named Inventor	Takada
Group Art Unit	1654
Examiner Name	Gupta, Anish
Attorney Docket No.	FJN-058C1
Patent No.	8309
Issue Date	Not applicable

ENCLOSURES *(check all that apply)*

- ☐ Fee Transmittal Form
  - ☐ Check Attached
  - ☐ Copy of Fee Transmittal Form
- ☐ Amendment/Response
  - ☐ Preliminary
  - ☐ After Final
  - ☐ Affidavits/declaration(s)
  - ☐ Letter to Official Draftsperson including Drawings [Total Sheets _____]
- ☐ Petition for Extension of Time
- ☐ Information Disclosure Statement
  - ☐ Form PTO-1449
  - ☐ Copies of IDS Citations
- ☐ Certified Copy of Priority Document(s)
- ☐ Sequence Listing submission
  - ☐ Paper Copy/CD
  - ☐ Computer Readable Copy
  - ☐ Statement verifying identity of above
- ☐ Copy of Notice to File Missing Parts of Application
- ☐ Formal Drawing(s)
- ☐ Request For Continued Examination (RCE) Transmittal
- ☐ Power of Attorney (Revocation of Prior Powers)
- ☒ Return Receipt Postcard
- ☒ Petition to Withdraw Holding of Abandonment under 37 C.F.R. § 1.181 (2 pgs.)
- ☐ Small Entity Statement
- ☐ CD(s) for large table or computer program
- ☐ Amendment After Allowance
- ☐ Request for Certificate of Correction
  - ☐ Certificate of Correction (in duplicate)
- ☒ Additional Enclosure(s) *(please identify below)*

Exhibit A: Copy of Return Receipt Postcard stamped September 9, 2004 (1 pg.)

Exhibit B: Copy of Express Mail Mailing Label EV375068968US stamped and dated September 9, 2004 (1 pg.)

Exhibit C: Copy of Amendment and Response (9 pgs.)

Exhibit D: Copy of Petition for Extension of Time Under 37 C.F.R. 1.136(a) (1 pg.)

Exhibit E: Copy of Check in the amount of $1130.00 (1 pg.)

Exhibit F: Copy of Supplemental Information Disclosure Statement (2 pgs.)

Exhibit G: Copy of Supplemental Form PTO-1449 (1 pg.)

Exhibit H: Copy of IDS Citation "CL" (18 pgs.)

Exhibit I: Copy of 'Exhibit A' in Support of Amendment and Response –"The Bioavailability of Dietary Calcium" (18 pgs.)

Exhibit J: Copy of Transmittal Form dated September 9, 2004 (1 pg.)

Exhibit K: Copy of Fee Transmittal Form dated September 9, 2004 (1 pg.)

Copy of Notice of Abandonment mailed September 21, 2004 (2 pgs.)

CORRESPONDENCE ADDRESS

Direct all correspondence to:

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Testa, Hurwitz & Thibeault, LLP
High Street Tower
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Boston, MA 02110
Tel. No.: (617) 248-7000
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SIGNATURE BLOCK

Brian Fairchild

Respectfully submitted,
on behalf of Christine Vito
Brian Fairchild, Reg. No. 48,645

Date: September 28, 2004
Reg. No. 39,061
Tel. No.: (617) 248-7368
Fax No.: (617) 248-7100

Christine C. Vito
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Express Mail Mailing Label No.: EV334240737US

PATENT
Attorney Docket No. FJN-058C1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT(S):	Takada *et al.*	CONF. NO.	8309
SERIAL NO.:	10/067,527	GROUP NO.	1654
FILING DATE:	February 4, 2002	EXAMINER:	Gupta, Anish
TITLE:	COMPOSITIONS FOR STRENGTHENING BONE		

Mail Stop Petition
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

PETITION TO WITHDRAW HOLDING OF ABANDONMENT UNDER 37 C.F.R. § 1.181

The above-referenced application was deemed abandoned for Applicants' failure to timely file a proper reply to the Office letter mailed on March 9, 2004.

The Notice of Abandonment mailed on September 21, 2004, erroneously states that no reply to the Office letter mailed on March 9, 2004, has been received by the United States Patent and Trademark Office.

Applicants submit that a timely and proper reply was in fact filed on September 9, 2004. As evidence thereof, Applicants submit herewith copies of all documents submitted in response to the Office letter of March 9, 2004. Applicants submit as "Exhibit A" a copy of the return receipt postcard stamped by the U.S. Patent and Trademark Office, indicating receipt of the below-listed items on September 9, 2004. Attached as "Exhibit B" is a copy of the Express Mail Mailing Label bearing the number EV375068968US on which the "Date In" is indicated as September 9, 2004; the United States Postal Service stamp also indicates the date of deposit as September 9, 2004. This Express Mail Mailing Label number is present as required on each paper of the submission of September 9, 2004. "Exhibit C" is a copy of the submitted Amendment

and Response (9 pages). "Exhibit D" is a copy of the submitted Petition for Three Months' Extension of Time Under 37 C.F.R. 1.136(a) (1 page). "Exhibit E" is a copy of the submitted check in the amount $1130.00. "Exhibit F" is a copy of the submitted Supplemental Information Disclosure Statement (2 pages). "Exhibit G" is a copy of the submitted Supplemental Form PTO-1449 (1 page). "Exhibit H" is a copy of reference "CL" as cited on the submitted Supplemental Form PTO-1449 (18 pages). "Exhibit I" is a copy of submitted article 'The Bioavailability of Dietary Calcium,' originally submitted as 'Exhibit A' in support of the Amendment and Response (18 pages). "Exhibit J" is a copy of the submitted Transmittal Form (1 page). "Exhibit K" is a copy of the submitted Fee Transmittal Form (1 page).

A copy of the Notice of Abandonment mailed September 21, 2004, is also enclosed. Applicants would like to draw attention to the Examiner's note at the bottom of the Notice which indicates that a Dr. Blecher was contacted regarding the instant application. Dr. Blecher is not an attorney of record in this case. Applicants believe this note is in error and do not assent to the facts stated therein.

In view of the evidence presented above, Applicants respectfully request that the abandonment be withdrawn.

Applicants believe no fees are due for entry of this petition. However, please consider this a conditional authorization to charge deposit account 20-0531 for any required fees or surcharges.

Respectfully submitted,

Brian Fairchild on behalf of Christine Vito
Brian Fairchild, Reg No. 48,645

Date: September 28, 2004
Reg. No. 39,061

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/067,527	02/04/2002	Yukihiro Takada	FJN-058C1	8309

21323 7590 09/21/2004

TESTA, HURWITZ & THIBEAULT, LLP
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BOSTON, MA 02110

EXAMINER	
GUPTA, ANISH	
ART UNIT	**PAPER NUMBER**
1654	

DATE MAILED: 09/21/2004

TESTA, HURWITZ & THIBEAULT, LLP
PATENT DEPARTMENT

Please find below and/or attached an Office communication concerning this application or proceeding.

Notice of Abandonment

Application No.	Applicant(s)	
10/067,527	TAKADA ET AL.	
Examiner	Art Unit	
Anish Gupta	1654	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

This application is abandoned in view of:

1. ☒ Applicant's failure to timely file a proper reply to the Office letter mailed on *09 March 2004*.
   (a) ☐ A reply was received on _____ (with a Certificate of Mailing or Transmission dated _____), which is after the expiration of the period for reply (including a total extension of time of _____ month(s)) which expired on _____.
   (b) ☐ A proposed reply was received on _____, but it does not constitute a proper reply under 37 CFR 1.113 (a) to the final rejection.
   (A proper reply under 37 CFR 1.113 to a final rejection consists only of: (1) a timely filed amendment which places the application in condition for allowance; (2) a timely filed Notice of Appeal (with appeal fee); or (3) a timely filed Request for Continued Examination (RCE) in compliance with 37 CFR 1.114).
   (c) ☐ A reply was received on _____ but it does not constitute a proper reply, or a bona fide attempt at a proper reply, to the non-final rejection. See 37 CFR 1.85(a) and 1.111. (See explanation in box 7 below).
   (d) ☒ No reply has been received.

2. ☐ Applicant's failure to timely pay the required issue fee and publication fee, if applicable, within the statutory period of three months from the mailing date of the Notice of Allowance (PTOL-85).
   (a) ☐ The issue fee and publication fee, if applicable, was received on _____ (with a Certificate of Mailing or Transmission dated _____), which is after the expiration of the statutory period for payment of the issue fee (and publication fee) set in the Notice of Allowance (PTOL-85).
   (b) ☐ The submitted fee of $_____ is insufficient. A balance of $_____ is due.
   The issue fee required by 37 CFR 1.18 is $_____. The publication fee, if required by 37 CFR 1.18(d), is $_____.
   (c) ☐ The issue fee and publication fee, if applicable, has not been received.

3. ☐ Applicant's failure to timely file corrected drawings as required by, and within the three-month period set in, the Notice of Allowability (PTO-37).
   (a) ☐ Proposed corrected drawings were received on _____ (with a Certificate of Mailing or Transmission dated _____), which is after the expiration of the period for reply.
   (b) ☐ No corrected drawings have been received.

4. ☐ The letter of express abandonment which is signed by the attorney or agent of record, the assignee of the entire interest, or all of the applicants.

5. ☐ The letter of express abandonment which is signed by an attorney or agent (acting in a representative capacity under 37 CFR 1.34(a)) upon the filing of a continuing application.

6. ☐ The decision by the Board of Patent Appeals and Interference rendered on _____ and because the period for seeking court review of the decision has expired and there are no allowed claims.

7. ☐ The reason(s) below:

A phone call was made to Dr. Blecher to see if a response had been filed to the outstanding office action. Dr. Blecher indicated a response had been filed via fax but he did not have any confirmation of sending out the response.

Bruce Campell

BRUCE R. CAMPELL, PH.D
SUPERVISORY PATENT EXAMINER
TECHNOLOGY CENTER 1600

Petitions to revive under 37 CFR 1.137(a) or (b), or requests to withdraw the holding of abandonment under 37 CFR 1.181, should be promptly filed to minimize any negative effects on patent term.

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The "RECEIVED" stamp of the United States Patent and Trademark Office imprinted hereon acknowledges the filing of:

Transmittal Form (1 page); Fee Transmittal Form (1 pg.); Check in the amount of $1130.00; Amendment and Response (9 pgs.); Exhibit A - "The Bioavailability of Dietary Calcium;" *Journal of American College of Nutrition*, Vol. 19(2), 119S-136S (2000) (18 pgs.); Petition for Extension of Time Under 37 C.F.R. 1.136(a) (1 pg.); Supplemental Information Disclosure Statement (2 pgs.); Supplemental Form PTO-1449 (1 pg.); Copy of Cited Reference (CL-18 pgs.); and this return receipt postcard, with each paper and fee bearing Express Mail Mailing Label No. EV375068968US.

Name of Applicant: Takada et al.

Serial Number: 10/067,527

Atty: CCVito/CAKomm

Date: September 9, 2004
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PATENT
Attorney Docket No. FJN-058CJ

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT(S):	Takada *et al.*	CONFIRMATION NO.:	8309
SERIAL NO.:	10/067,527	GROUP NO.:	1654
FILING DATE:	February 4, 2002	EXAMINER:	Gupta, Anish
TITLE:	Compositions for Strengthening Bone		

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

AMENDMENT AND RESPONSE

Sir:

In response to the Office Action mailed March 9, 2004, in connection with the above-identified patent application, Applicants respectfully submit the following Amendment and Response. A Petition for a 3-month extension of time, up to and including September 9, 2004, as well as a Supplemental Information Disclosure Statement (SIDS) is submitted herewith. Also enclosed is a check for $1130.00 to cover the $950.00 petition fee and the $180.00 SIDS submission fee. If an additional fee is required, please consider this a conditional petition therefore, and authorization to debit deposit account 20-0531.

Amendments to the Claims are presented on page 2 of this paper.

Remarks/Argument begin on page 4 of this paper.

AMENDMENTS TO THE CLAIMS

1-48. (Canceled)

49. (Currently Amended) A composition for strengthening bone in a mammal comprising degraded collagen, calcium, and vitamin D_3 wherein said composition is suitable for oral administration in an amount effective to induce a bone strengthening effect in a mammal.

50. (Previously Presented) The composition of claim 49, wherein the composition comprises enzymatically-degraded collagen.

51. (Previously Presented) The composition of claim 50, wherein the composition comprises collagen degraded by limited acid proteolysis.

52. (Previously Presented) The composition of claim 50, wherein the composition comprises collagen degraded by limited alkaline proteolysis.

53. (Currently Amended) The composition of claim 49, wherein the ~~composition comprises~~ the amount effective to induce a bone strengthening effect in a mammal is between 10 mg to 2500 mg of degraded collagen.

54. (Currently Amended) The composition of claim 49, wherein the weight ratio of collagen to calcium is between 0.5-5.0: 1.

55. (Currently Amended) The composition of claim 49, wherein the weight ratio of collagen to calcium is about 3.75: 1.

56 (Previously Presented) The composition of claim 49, wherein the calcium is selected from the group consisting of calcium chloride, calcium carbonate, calcium lactate, and egg-shell derived calcium, and milk-derived calcium.

57. (Previously Presented) The composition of claim 49, wherein the collagen is derived from porcine skin or bone.

58. (Previously Presented) The composition of claim 57, wherein the collagen derived from porcine skin is a lyophilization product of pulverized and defatted skin corium layer.

59. (Previously Presented) The composition of claim 57, wherein the wherein the collagen derived from porcine bone is a lyophylization product of pulverized and decalcified bone.

60. (Previously Presented) The composition of claim 49, wherein the degraded collagen has a molecular weight of about 2-150 kDa.

REMARKS

Claims 49-60 were pending in the Application. Claims 49 and 53-55 are amended herein. Upon entry of the present Amendment, claims 49-60 will be pending and are presented for reconsideration.

Claim 49 is amended to recite a composition for strengthening bone in a mammal comprising degraded collagen, calcium, and vitamin D_3 wherein said composition is suitable for oral administration in an amount effective to induce a bone strengthening effect. Claim 53 is amended to recite that the amount effective to induce a bone strengthening effect in a mammal is between 10 mg to 2500 mg of degraded collagen. Claims 54 and 55 have been amended to clarify the recited ratios. Support for these amendment can be found throughout the application as originally filed. Applicants respectfully submit that no new matter is introduced by this amendment.

Applicants note that while claim 56 was listed as rejected on Form PTOL-326 accompanying the Office action, no specific rejection was enumerated in the written Office action. Applicants therefore do not acquiesce to the rejection of claim 56.

Rejection Under 35 U.S.C. § 102(b)

Claims 49-52 and 57-60 were rejected under 35 U.S.C. § 102(b) as being allegedly anticipated by U.S. Patent No. 5,002,760 to Katzev (hereinafter "Katzev), in light of U.S. Patent No. 5,057,502 to Walsh (hereinafter "Walsh"). Applicants traverse the rejection.

For anticipation under 35 U.S.C. § 102, the reference must teach every aspect of the claimed invention either explicitly or impliedly. MPEP 706.02. Applicants submit that the Office action's rejection is improper because Katzev does not disclose every aspect of Applicants' invention as claimed herein.

Firstly, Katzev's invention is a topical skin care composition formulated for the sole and explicit purpose of maximizing retinol's effectiveness on skin, whereas Applicants' invention claims a composition suitable for oral administration in an amount effective to induce a bone strengthening effect. While Katzev allegedly teaches vitamin D_3, calcium, and degraded collagen, these are only three of over twenty ingredients in Katzev's composition. Katzev neither

teaches or suggests that these three specific ingredients could stand alone or be extracted from the Katzev topical composition for use alone or in combination for purposes unrelated to retinol delivery. Moreover, Katzev provides no indication that the quantities of calcium, hydrolyzed collagen, and vitamin D_3 in his composition would be capable of inducing a bone strengthening effect, as required by the claimed invention. Consequently, since Katzev does not teach the Applicants' claimed composition and effect, Katzev cannot anticipate the claimed invention.

Secondly, the Office action suggests that Katzev's composition contains calcium because aloe vera contains calcium oxalate. However, Applicants submit that Katzev is an improper reference because it does not teach the calcium of the claimed invention. Applicants' specification teaches that a "calcium agent with good absorptivity, such as calcium chloride, calcium carbonate, calcium lactate, egg shell or milk-derived calcium etc." can be used according to the invention to enhance the strengthening of bone. (See page 6, lines 5-8). While this is not an exhaustive list of calcium sources with good absorptivity, it is well known in the art that calcium oxalate is not an absorbable source of calcium. For example, in the article *The Bioavailability of Dietary Calcium* (attached as "Exhibit A"), it states, "Other dietary factors make calcium irreversibly insoluble at near-neutral pH values, by converting it into forms such as phosphates, oxalates, phytates and soaps, which prevent passive absorption in the ileum." *Journal of the American College of Nutrition, Vol. 19, No.2, 119S-136S (2000) at 121S.* This evidence clearly demonstrates that calcium oxalate, as found in the aloe vera of Katzev's composition, would not be absorbed by the body and therefore is not within the scope of Applicants' claimed invention.

Therefore, in light of the foregoing reasons, Applicants request reconsideration and withdrawal of the rejection of independent claim 49 and dependent claims 50-52 and 57-60 under 35 U.S.C. § 102(b).

Rejections Under 35 U.S.C. § 103(a)

Sauk et al. and Borenstein et al.

Claims 49-55 and 57-60 were rejected under 35 U.S.C. § 103(a) as being allegedly unpatentably obvious over U.S. Patent No. 4,698,326 to Sauk et al. (hereinafter "Sauk") in view of U.S. Patent No. 5,043,170 to Borenstein et al. (hereinafter "Borenstein"). Claim 49 as amended, and those claims depending therefrom (50-55 and 57-60), recite a composition comprising degraded collagen, calcium, and vitamin D_3 suitable for oral administration in an amount effective to induce a bone strengthening effect. The Office action suggests that Sauk teaches a composition for osseous repair made of phosphophoryn calcium salt and type I collagen. The Office action further suggests that Borenstein teaches that certain vitamin D_3 metabolites help increase bone strength in hens. The Office action suggests that it would have been obvious to one of ordinary skill in the art to use vitamin D_3 in conjunction with the composition of Sauk to produce the applicants' claimed invention because one would expect that the addition of Vitamin D_3 to the composition would result in a more effective bone strengthening or growing agent. Applicants respectfully traverse the rejection.

Applicants submit that, in fact, the deficiencies of Sauk cannot be remedied by the addition of Borenstein because there is no motivation to modify Sauk to incorporate the vitamin D_3 of Borenstein. Sauk teaches a method of locally promoting osseous growth in guinea pigs by surgically implanting a mixture of collagen and phosphophoryn calcium into an osseous void, whereas Borenstein teaches a method of improving eggshell strength in hens soley by oral administration of vitamin D_3 metabolites. Even though Borenstein mentions that vitamin D_3 is related to bone strength, Borenstein does not teach vitamin D_3 *per se* as an effective agent for promoting localized osseous growth. Because Borenstein's efforts to promote eggshell and bone strength through an orally administered composition are unrelated to Sauk's purpose of promoting localized osseous growth by direct implantation, a person skilled in the art would not be motivated to modify Sauk by the teachings of Borenstein to produce the Applicant's claimed invention.

Furthermore, U.S. Patent 4,335,120 to Holick et al. (hereinafter "Holick"), cited by the Office action, teaches away from combining Sauk and Borenstein. Holick explicity discourages

oral administration of vitamin D_3, stating that there is "a danger in administering biologically active vitamin D_3 orally or intravenously to patients [because] the therapeutic to toxic ratio of vitamin D_3 is low and an excess...in the blood stream can cause episodes of hypercalcemia and hypercalcuria". (Column 1, lines 26-31). Considering that the claimed invention requires a composition suitable for oral administration, a skilled artisan, on reading Holick, would be taught away from combining the vitamin D_3 of Borenstein with the composition of Sauk to produce the Applicants' claimed invention. Therefore, it is improper to combine the teachings of Sauk and Borenstein.

However, even if there were a motivation to combine these references, a person skilled in the art would have no reasonable expectation of success in producing the claimed invention. As the claims recite, the composition must be suitable for oral administration in an amount effective to induce a bone strengthening effect. However, while Sauk teaches a composition for local surgical implantation into an osseous void which is effective at inducing localized bone growth, there is no teaching in either Sauk or Borenstein to suggest that the combination of collagen, phosphophoryn calcium, and vitamin D_3 together would have a systemic bone strengthening effect if orally administered. Therefore, a skilled artisan would have no reasonable expectation that combining the teachings of Sauk and Borenstein would in fact result in the Applicants' claimed invention. Consequently, no *prima-facie* case of obviousness can be made.

Therefore, in light of the foregoing reasons, Applicants respectfully request that the rejection of independent claim 49 and dependent claims 50-55 and 57-60 under 35 U.S.C. § 103(a) be reconsidered and withdrawn.

Milan in view of Holick and Kato

Claims 49-55 and 57-60 were rejected under 35 U.S.C. 103(a) as being allegedly unpatentably obvious over U.S. Patent 5,948,766 to Milan et al. (hereinafter "Milan") in view of Holick (discussed *supra*), and U.S. Patent No. 5,932,259 to Kato et al. (hereinafter "Kato"). In particular, the Office action suggests that Milan discloses a method of treating osteoporosis with collagen and calcium salts. It further suggests that Kato teaches that specific calcium sources, as well as vitamin D_3, can be used to treat osteoporosis. It also suggests that Holick teaches that

vitamin D_3 is effective in increasing serum calcium for treating osteoporosis. Applicants traverse the rejection.

Applicants submit that there is no motivation to modify the teachings of Milan by the teachings of Kato. While Milan teaches that a preparation of hydrolyzed collagen and calcium salts can be used to treat osteoporosis, Kato explicitly teaches that there are various problems associated with using calcium and vitamin D_3 to strengthen bones. (See Col 1, lines 36-64). Kato teaches that "more than half of the calcium salts and natural calcium administered are excreted without being absorbed", and that whatever calcium is "absorbed may not necessarily be utilized for the improvement of bone metabolism or bone reinforcement because the affinity of calcium for bone differs according to the form of the calcium and types of other nutrients which are taken with calcium." (Column 1, lines 43-50). Kato also teaches that adding vitamin D_3 to food or drink is infeasible due to safety and cost considerations. (See Column 1, lines 50-58). Because Kato discourages use of calcium or vitamin D_3 in a composition to strengthen bone, a person skilled in the art would be taught away from adding calcium or vitamin D_3 to the composition of Milan to produce the claimed invention. Therefore, Kato cannot cure the deficiencies of Milan.

Furthermore, Applicants submit that there is no motivation to modify Milan by the teachings of Holick. While Milan teaches that a preparation of hydrolyzed collagen and calcium salts can be used to treat osteoporosis, it does not teach vitamin D_3 for such a purpose. In fact, Holick teaches that there is "a danger in administering biologically active vitamin D_3 orally or intravenously to patients [in] that the therapeutic to toxic ratio of vitamin D_3 is low and an excess... in the blood stream can cause episodes of hypercalcemia and hypercalcuria". (Column 1, lines 26-31). To avoid this hazard, Holick teaches that vitamin D_3 should be administered on or into the skin. (See Column 1, lines 54-59). Therefore, Holick teaches away from oral administration of vitamin D_3. Considering that Milan's composition of collagen and calcium is "preferably formulated as pastes, syrups, solutions, granules, compressed formulations or instantized powders," (Column 2, lines 48-53) and that patients in Milan received a "diet rich in collagen hydrolysate," (Column 3, lines 13-17), it is clear that Milan's collagen composition is delivered orally. In view of the claimed invention's requirement that the composition be suitable for oral administration, and because Holick discourages oral administration of vitamin D_3, a

person skilled in the art would be taught away from adding vitamin D_3 to Milan's orally administered composition to produce the applicants' claimed invention. Therefore, Holick cannot cure the deficiencies of Milan.

In light of the foregoing reasons, Applicants respectfully request that the rejection of independent claim 49 and dependent claims 50-55 and 57-60 under 35 U.S.C. § 103(a) be reconsidered and withdrawn.

CONCLUSION

Claims 49-60 were pending in the Application. Claims 49 and 53-55 are amended. Applicants respectfully submit that no new matter is introduced by the present Amendment.

Applicants request that the Examiner reconsider the application in light of the foregoing Amendment and Response, and respectfully submit that the claims are in condition for allowance. If the Examiner believes a telephonic interview would expedite the favorable prosecution of this application, the undersigned attorney would welcome the opportunity to discuss any outstanding issues, and to work with the Examiner toward placing the application in condition for allowance.

Respectfully submitted,

Date: September 9, 2004
Reg. No. 39,061

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PETITION FOR EXTENSION OF TIME UNDER 37 CFR 1.136(a)	Attorney Docket Number FJN-058C1

In re Application of Takada *et al.*	
Application Serial No. 10/067,527	
Filed: February 4, 2002	
Group Art Unit: 1654	Examiner: Gupta, Anish

This is a request under the provisions of 37 CFR 1.136(a) to extend the period for filing a response in the above entitled application.

The requested extension and appropriate non-small-entity fee are as follows (check time period desired)

☐	One month (37 CFR 1.17(a)(1))	$
☐	Two months (37 CFR 1.17(a)(2))	$
☒	Three months (37 CFR 1.17(a)(3))	$ 950.00
☐	Four months (37 CFR 1.17(a)(4))	$
☐	Five months (37 CFR 1.17(a)(5))	$

☐ Applicant is a small entity under 37 CFR 1.9 and 1.27, therefore the fee amount shown above is reduced by one-half, and the resulting fee is: $___.
A small entity statement under 37 CFR 1.27:

☒ A check in the amount of $1130.00 is enclosed, $950.00 of which is for the petition fee.

☐ The Commissioner is hereby authorized to charge the required fee to Deposit Account No. 20-0531. Enclosed is a copy of this sheet.

☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment, to Deposit Account No. 20-0531.

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANTS:	Takada *et al.*	CONFIRMATION NO.:	8309
SERIAL NO.:	10/067,527	GROUP NO.:	1654
FILING DATE:	February 4, 2002	EXAMINER:	Gupta, Anish
TITLE:	Compositions For Strengthening Bone		

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Commissioner for Patents
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Alexandria, VA 22313-1450

SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT

Sir:

In accordance with the provisions of 37 C.F.R. 1.97 and 1.98, Applicants hereby make of record the publication listed on the accompanying Form PTO-1449, and other information contained herein, for consideration by the Examiner in connection with the examination of the above-identified patent application. A Copy of the publications is enclosed.

REMARKS

In accordance with the provisions of 37 C.F.R. 1.97, this statement is being filed (CHECK ONE):

☐ (1) within three (3) months of the **filing date** of a national application other than a continued prosecution application under 37 C.F.R. 1.53(d), or within three (3) months of the **date of entry of the national stage** as set forth in 37 C.F.R. 1.491 in an international application, or before the mailing of the **first Office action** on the merits, or before the mailing of a **first Office action** after the filing of a request for continued examination under 37 C.F.R. 1.114; or

☒ (2) after the period defined in (1) but before the mailing date of a **final action** or a **notice of allowance** under 37 C.F.R. 1.311, and

☐ the requisite Statement is below, **OR**

☒ the requisite fee under 37 C.F.R. 1.17(p), namely **$180.00**, is included herein, or

☐ (3) after the mailing date of a **final action** or **notice of allowance** but before the payment of the **issue fee**, **AND**

☐ the requisite Statement is below, **AND**

☐ the requisite petition fee under 37 C.F.R. 1.17(p), namely **$180.00** is included herein.

It is respectfully requested that each of the patents and publications listed on the attached Form PTO-1449, and other information contained herein, be made of record in this application.

Respectfully submitted,

Date: September 9, 2004
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FORM PTO - 1449 SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT	ATTY DOCKET NO.: FJN-058CI APPLICANT: Takada et al. SERIAL NO.: 10/067,527 FILING DATE: February 4, 2002 GROUP: 1653 CONFIRMATION NO.: 8309

U.S. PATENT DOCUMENTS

EXAM. INIT.		DOCUMENT NUMBER	DATE	NAME	CLASS	SUB CLASS	FILING DATE IF APPROPRIATE

FOREIGN PATENT DOCUMENTS

EXAM INIT.		DOCUMENT NUMBER	DATE	COUNTRY CODE	CLASS	SUB CLASS	FILING DATE	ABSTRACT ONLY	ENGLISH LANG Y/N

OTHER ART, JOURNAL ARTICLES, ETC.

EXAM. INIT.	OTHER DOCUMENTS: (Including Author, Title, Date, Relevant Pages, Place of Publication)	
	CL	Guéguen et al., "The Bioavailability of Dietary Calcium," *Journal of American College of Nutrition*, Vol. 19(2), 119S-136S (2000).

EXAMINER	DATE CONSIDERED

3115912

The Bioavailability of Dietary Calcium

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Key words: calcium, bioavailability, humans, milk, absorption, bone

This update focuses on the bioavailability of dietary calcium for humans. Fundamentals of calcium metabolism, intestinal absorption, urinary excretion and balance are recalled. Dietary factors, especially lactose and other milk components, influencing calcium bioavailability at intestinal and renal levels are reviewed. A critical examination of all the methods used for evaluating calcium bioavailability is made. This includes *in vitro* assays, classical and isotopic balances, urinary excretion, isotope labeling in the urine, plasma and bones, long-term evaluation of bone mineralization and the use of biological bone markers. Importance and advantages of animal models are discussed. The state of the art in the comparative bioavailability of calcium in foods is detailed including a comparison of sources of calcium (dairy products and calcium salts) in human studies and in some animal studies, casein phosphopeptides, proteins, lactose and lactase and their relation with calcium bioavailability (in humans and rats). An update on the consumption of dairy products and bone mass is presented. Emphasis on peculiarities and advantages of calcium in milk and dairy products is given.

Key teaching points:

- Milk provides large amounts of calcium and phosphorus and components such as lactose and casein phosphopeptides which may enhance calcium absorption and mineral retention.
- Using a variety of methods, no one has shown that the calcium in milk is more efficiently absorbed that most calcium salts.
- Intestinal absorption does not necessarily reflect the bioavailability of calcium to the whole organism because calcium must be retained and used in bone formation and mineralization.
- Three sources of calcium, milk, calcium carbonate and calcium citromalate, have been extensively studied. They all ensure the efficient absorption of calcium and also ensure, over the long term, that calcium is retained and used for bone mineralization.
- There is, as yet, no evidence showing that the calcium from mineral water is as effective.
- Many direct or indirect methods may be used to evaluate calcium bioavailability. The values obtained depend on the method; thus, conclusions or comparisons must be drawn with care.

INTRODUCTION

Both scientists and the general public are becoming increasingly aware of the importance of dietary calcium. This is largely due to the many research studies that have demonstrated links between dietary calcium intake and diseases such as osteoporosis, arterial hypertension and colon cancer. These diseases have many causes, but the scientific community now recognizes that dietary calcium helps prevent them.

The average recommended dietary allowance (RDA) or adequate intake (AI) of calcium is about 900 mg per day (800 to 1000 mg, depending on the country) for adults, rising to 1200 mg/day for adolescents and the elderly. These RDAs are safety levels designed to provide adults with maximum protection against a negative calcium balance and, hence, against bone loss. But they are also set so as to ensure that adolescents produce the maximum amount of bone that is genetically possible and, hence, remain above the fracture threshold when they become older.

A recent review of calcium consumption in France [1] determined the percentage of each sector of the population that consumed less than two thirds of the RDA, the critical threshold for defining groups at risk. These groups included about 20% to 25% of men aged 18 to 65, 30% of women aged 18 to 50, 50% of adolescent girls and men aged over 65, and 75% of women over the age of 55. Elderly women living in institutions had particularly low calcium intakes.

About 70% of dietary calcium comes from milk and dairy products, mainly cheese in adults. Only a few green vegetables and dried fruits are good sources of calcium (16% of

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intake), and drinking water, including mineral water, provides 6% to 7%.

There is no doubt that milk provides large amounts of calcium. While there is also no question of the nutritional effectiveness of the calcium provided by milk, there is still some debate as to whether this source of calcium is biologically better than other sources, such as calcium salts, certain vegetables or mineral waters. We have therefore included recent publications in which the calcium provided by dairy products is compared to calcium from these other sources. While our coverage is more extensive than that of many other reviews, there has been so much work published on this topic that we cannot claim to have cited all data. For general aspects of calcium metabolism and factors influencing bioavailability, we have drawn extensively on our earlier reviews of calcium availability [2,3] and complementary data may be found in other reviews [4–6].

The review focuses particularity on the bioavailability of calcium from milk and dairy products.

DEFINITIONS AND WAYS OF EXPRESSING BIOAVAILABILITY

Absorbability, or the availability of calcium for absorption by the intestines, is often used as a synonym for bioavailability. It is, however, no more than the first step towards bioavailability. Calcium must be soluble in the acid medium of the stomach before it can be absorbed. Good solubility in water is an advantage but is not absolutely necessary. The intestinal absorption values measured in humans and animals are not always equivalent to, and are generally lower than, calcium absorbability. The potential absorbability of calcium depends on the food, whereas absorption depends also on the absorptive capacity of the intestines, which is affected by physiological factors such as calcium reserves, hormonal regulation or previous dietary calcium supply. The potential absorbability is thus the absorption under the most favorable physiological conditions.

Bioavailability depends on absorbability and the incorporation of absorbed calcium into bone. Hence, it also depends on the urinary excretion and fecal loss of endogenous calcium. As for intestinal absorption, physiological factors, particularly hormones, play a major role in the incorporation of calcium into bone. However, certain types of food increase the likelihood that absorbed calcium will be incorporated into bone, whereas others result in calcium being mainly excreted in the urine. The effects of small changes in the diet on the net calcium balance have been emphasized by several studies. Thus, certain anions, such as sulfate and chloride, organic ligands (chelators) and excess protein or sodium all increase the loss of calcium in the urine and, thus, hinder its incorporation into bone. Conversely, the incorporation of absorbed calcium into bone is stimulated by phosphorus, but excess phosphorus may also cause undesirable ectopic calcification (outside of the bone). The bioavailability of calcium may therefore be defined as the fraction of dietary calcium that is potentially absorbable by the intestine and can be used for physiological functions, particularly bone mineralization, or to limit bone loss.

Absorbability and bioavailability may be absolute or relative. Unless defining dietary needs by the factorial method, relative values are sufficient for determining the fraction absorbed in comparison with different sources of calcium. The values are then expressed relative to a reference source.

The values measured may be mean values or discrete values. Mean values are recorded for a whole diet or a single source of calcium studied over a period of weeks or months after adaptation. Discrete values are for a single meal or a single oral calcium load. They correspond less to normal dietary conditions than mean values and do not take into account the large variations that occur over time.

FUNDAMENTALS OF CALCIUM METABOLISM

Intestinal Absorption

Calcium must be in a soluble form, generally ionized (Ca^{++}), at least in the upper small intestine or bound to a soluble organic molecule before it can cross the wall of the intestine. Absorption is the result of two processes, active transport across cells, mainly in the duodenum and the upper jejunum, and passive diffusion, which occurs throughout the small intestine, but mainly in the ileum [7] and very little in the large intestine [8].

Active Transport. The active transport system for calcium is saturable and regulated by dietary intake and the needs of the body. It involves three stages: entry across the brush border of the enterocyte, diffusion across the cytoplasm and secretion across the basolateral membrane into the extracellular liquid [9,10].

Calcium enters the cell via a positive electrochemical gradient because the calcium concentration in the cytoplasm is very low. It crosses the membrane via calcium channels and via membrane-binding transport proteins (calmodulin and membrane calcium-binding proteins). It may be stored transiently in organelles like the Golgi apparatus, endoplasmic reticulum (ER) or mitochondria, but then crosses the cytoplasm attached to a calcium binding protein (CaBP or calbindin-D 9K), which is the rate limiting factor in active calcium transport. Calcium may travel bound to the protein if the CaBP remains in the cytoplasm or via membrane-bound vesicles if the CaBP is incorporated into the lysosomes [9]. It is extruded from the cell against an electrochemical gradient by two routes. A small fraction leaves by exchanging 3 Na^+ for 2 Ca^{++}, but most leaves via a calcium pump, a Ca-ATPase activated by calcium, CaBP and calmodulin.

Vitamin D influences several steps in this active transport. The active metabolite is 1,25 dihydroxycholecalciferol ($1,25(OH)_2D_3$ or calcitriol), which is produced by two hydroxylations of vitamin D, one in the liver (at position 25) and the other in the kidney (at position 1). These reactions occur whether vitamin D3 comes from the diet or from UV irradiation of 7-dehydrocholesterol in the skin. The most striking effect of calcitriol is its control of the expression of the gene encoding CaBP, causing the synthesis of the protein, thereby regulating the migration of calcium across intestinal cells. Calcitriol also has a "liponomic" action, increasing membrane permeability and activating the Ca-ATPase [9–11]. Calcitriol behaves like a hormone. Its renal production is regulated by parathyroid hormone (PTH), the secretion of which is, in turn, stimulated by a fall in plasma calcium concentration, which may itself stimulate calcitriol synthesis. The PTH-calcitriol system is also involved in bone resorption and increases the reabsorption of calcium by the renal tubule. This hormone system therefore controls all the calcium that enters the extracellular pool of exchangeable calcium and ensures that the plasma calcium concentration varies little from 100 mg/L.

The rate of saturable, physiologically regulated active absorption is negatively correlated with dietary calcium intake. Newborn babies lack this active process, and old animals (most studies have been done on rats) have calcitriol receptors, but they are less abundant than in younger animals and the renal 1-alpha-hydroxylase is less active; this is also the case in elderly people [12].

Supplementing the diet with vitamin D is not always allowed (it is forbidden in France), so most vitamin D comes from UV irradiation of the skin. However, the recommended daily dietary intake of vitamin D for adults is about 400 IU (10 micrograms).

Some of the membrane and cytosolic proteins involved in calcium transport are not vitamin D-dependent. One such protein is calmodulin, and others may be dietary proteins like alpha lactalbumin, which may act like calmodulin [9]. Apart from vitamin D deficiency, these are the only dietary means of affecting this highly regulated physiological route of calcium absorption.

Passive Diffusion. Passive absorption down an electrochemical gradient occurs via intercellular junctions or spaces. It involves the mass movement of water and major solutes such as sodium and glucose. It is not saturable and therefore increases with dietary intake, provided that the calcium in the intestines is in an absorbable form. It is independent of vitamin D and age [9–11].

All components of the diet that make calcium soluble or keep it in solution within the ileum should stimulate passive diffusion. Several molecules do this, particularly milk proteins like the phosphopeptides derived from casein [13,14] and amino acids like L-lysine and L-arginine, which form soluble chelates with calcium [10]. Lactose and other carbohydrates, which are gradually absorbed, also have an effect but the mechanism involved is still a matter of controversy. It is now generally agreed that lactose, at least in high doses, increases the passive absorption of calcium in the absence of vitamin D and, consequently, decreases intestinal CaBP concentration and active transport of calcium [15].

All molecules that increase the osmolarity of the liquid in the ileum are likely to stimulate the passive diffusion of calcium [15], whereas certain amino acids act on the intercellular space causing contraction of the cytoskeleton [11].

Other dietary factors make calcium irreversibly insoluble at near-neutral pH values, by converting it into forms such as phosphates, oxalates, phytates and soaps, which prevent passive absorption in the ileum. A variety of dietary factors have been shown to affect the passive diffusion of calcium, and this is a promising area of research aimed at producing the "extra" absorption that is generally desired.

Excretion, Retention and Balance of Absorbed Calcium

Most retained calcium is stored in the skeleton (99% of the body's calcium), depending on its needs. The main factors affecting the efficiency of calcium storage in bone are not dietary; they are physiological, related to growth, pregnancy and lactation, for example. The deposition and resorption of bone are regulated by several hormones (e.g., PTH, calcitonin, calcitriol and estrogens), the actions of which are outside the scope of this review. Excess absorbed calcium that cannot be stored in bone is excreted in urine, feces and sweat. The calcium balance in adult humans is zero, so all absorbed calcium is excreted by these routes, possibly after being incorporated into and then released from bone.

Almost all the calcium reabsorbed by the intestinal tract comes from secretions like the bile, and the endogenous calcium excreted in feces is the fraction that is not reabsorbed.

The urinary loss results from glomerular filtration (about 10g Ca per day) and tubular reabsorption, which retrieves over 98% of the filtered load [16]. Like intestinal absorption and bone exchange, the renal calcium flux is regulated by hormones, tubular reabsorption being particularly tightly regulated by PTH.

The amounts of calcium in human urine are much larger than those in the urine of other animals. Changes in the amount of calcium excreted in the urine may therefore have a major impact on calcium balance [17]. Certain dietary factors can influence the tubular reabsorption of calcium (see below), and these must be carefully noted when evaluating calcium bioavailability.

Fig. 1 shows the main pathways of calcium in adult humans. Human adults lose approximately 0.3% of their bone mass each year; this means that their calcium balance is negative and they lose about 10 mg of calcium each day. This loss of bone mass may be ten times greater in post-menopausal women.

The ultimate goal of all hormonal regulation of intestinal

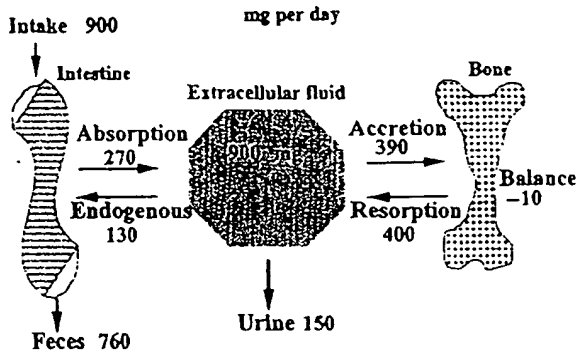

Fig. 1. The main pathways of calcium in adult humans.

absorption, bone resorption and renal tubular reabsorption of calcium is to keep the plasma calcium concentration constant, particularly the 50% of calcium in the ionic form. PTH and calcitriol are the most important hormones in calcium homeostasis. This complex control mechanism also regulates extracellular calcium, of which there are about 900 mg in the human body. Extracellular fluid (ECF) contains about 10^{-3} M Ca^{++}; the concentration of calcium ions in the cytosol is more than a 1000 times lower [16].

DIETARY FACTORS INFLUENCING CALCIUM BIOAVAILABILITY

This review covers only exogenous factors associated with the diet. Other endogenous factors like age, physiological condition and hormonal regulation have been discussed earlier [4–6,17] and are not discussed here. The main cause of changes in the rate of absorption and retention of calcium is clearly dietary intake, and there is an inverse relationship between intake and utilization. These changes have little to do with potential bioavailability, which is not controlled by hormones and does not reflect the absorptive capacity of the intestines or the retentive capacity of bone.

Dietary Factors Influencing Intestinal Absorption

Some components of the diet, such as the phytates found in bran and most cereals and seeds, oxalates in spinach, rhubarb, walnuts and sorrel, and tannins (tea), can form insoluble complexes with calcium, thereby reducing its absorbability. This only seems to affect calcium balance if the diet is unbalanced, high-fiber strict vegetarian diets lacking dairy products (calcium), for example. This must be taken into account when comparing dairy products with soybean-based products, which are generally phytate-rich. The apparently negative influence of fiber on calcium absorption is mainly due to the phytates that are frequently associated with dietary fiber. Other plant compounds, lightly methoxylated pectins for example, strongly inhibit the absorption of calcium and other minerals [18]. Fibers themselves (cellulose, hemicelluloses, lignins and non-cellulose polysaccharides) seem to have no direct effect on calcium absorbability. Some indigestible carbohydrates and hard-to-digest oligosaccharides have been shown to increase calcium absorption in the distal intestine by enhancing bacterial fermentation, thereby lowering the pH [19]. The effect of fibers and phytates has been examined in several reviews [e.g., 4,20]. A relative excess of phosphate has been thought to increase the fecal excretion of calcium. However, contrary to this widely held view, excess P does not reduce calcium absorption, at least if calcium intake is adequate. All Western-type meals have a Ca/P ratio well below 1, which favors the precipitation of calcium. This does not, however, prevent the normal absorption of calcium. Furthermore, the calcium in calcium phosphate is as well absorbed as the calcium in other inorganic salts, whether eaten with or without lactose [21].

Lipids, especially milk fats, are thought by some to form insoluble soaps with calcium, reducing its bioavailability. However, although this chemical reaction is possible, it does not, in practice, interfere with calcium absorption [4]. The dietary soaps are dissociated at the low pH of the stomach and cannot reform until they reach the ileum, which is beyond the main area of calcium absorption. Fecal soaps are formed from free long-chain saturated fatty acids and unabsorbed calcium. The saturated fatty acids in milk and cheese can displace calcium from phosphates in the ileum, forming less soluble soaps which are excreted, but this has no effect on the absorption of ingested calcium [22].

Other constituents of food, particularly components of milk, are thought to favor the intestinal absorption of calcium and to keep it in a soluble form until it reaches the distal intestine, where it can be absorbed by unsaturable routes that are independent of vitamin D. The best known are lactose, proteins and phosphopeptides.

Many *in vivo* and *in vitro* studies on proteins and phosphopeptides have demonstrated a positive effect of these molecules on calcium absorption. Phosphopeptides, derived from the enzymatic hydrolysis of caseins in particular, have been shown to sequester calcium and other cations, protecting them from potentially precipitating anions like phosphates in the intestine [13,14,23]. Phosphopeptides therefore help to keep calcium in solution until it reaches the distal intestine, thereby facilitating its absorption by passive diffusion.

Whey proteins, such as alpha lactalbumin and beta lactoglobulin, also bind calcium. Alpha lactalbumin binds calcium very tightly, making it a true binding protein, like calmodulin. However, despite the sometimes spectacular effects of these proteins and peptides on the solubility of calcium in the intestines *in vitro*, they have a much less dramatic effect on calcium absorption and retention *in vivo* [3].

The beneficial effect of lactose on the absorption of calcium and other cations has been more intensively studied than the effects of any other components of milk since it was demonstrated in rats by Bergheim in 1926 [24]. Interest increased

following the studies of French [25–27] and American [28–32] groups in the 1950s on the "lactose effect". The scientific debate on this issue is well described in the review by Miller [5], which is very well documented, but still incomplete.

It was first thought by the group of Fournier that lactose and other "structural" sugars acted directly on bone like precursors of bone proteins. This notion was replaced by theories of an intestinal action [7,28,29]. It is now clear that lactose, like other slowly absorbed sugars, must be at the site of absorption [28], that it prolongs the passive, vitamin D-independent absorption of calcium in the ileum [5,33] and that the effects of this action may be spectacular (doubling absorption) if a high dose of lactose (15% to 30% of the diet) is given.

Several theories have stressed the importance of keeping calcium soluble in the distal part of the intestines by forming soluble chelates [34] or by competition with inhibitors, such as phosphates. Fournier *et al.* [26] studied the effect of competition between lactose and phosphate on calcium absorption: lactose, like any other sugar that can be phosphorylated, accepts a phosphate group in a reaction catalyzed by alkaline phosphatase, thereby reducing the inhibition by phosphates within the lumen of the intestines. These authors therefore provided an explanation of why lactose in milk has little effect: the lactose and phosphate in milk have opposing effects.

It has been shown, however, that lactose does not act by increasing the concentration of soluble calcium in the lumen or by increasing the solubility of calcium phosphate *in vitro* [30]. There is also no cotransport of lactose and calcium [32]. The American group supported the idea that lactose acts on the intestinal mucosa to increase its permeability. All high osmolarity solutions double or triple the passive diffusion of calcium, probably by increasing the space of the intercellular junctions. This simple explanation may account for the effect of high doses of lactose [11]. Other studies [7,35] have shown that lactose and other sugars increase the absorption of calcium in the jejunum proportionally to their effects on water and sodium absorption.

The reduced bone resorption leads to the inhibition of bone turnover [27] in rats fed a lactose-enriched diet. This is caused by a large increase in intestinal calcium absorption [5]. However, the rat is not the appropriate model in which to study human bone remodeling (see below).

The effect of lactose has been clearly demonstrated in many experiments *in vitro* and in short- and long-term trials in rats, but its significance for human nutrition is much less clear [5,21]. Paradoxically, lactose, at least at physiological concentrations, does not seem to significantly affect the absorption of calcium from milk [36,37]. Only very high doses of lactose (50 g/day) have a net effect [7,38]. Calcium from yogurt, in which lactose is partially hydrolyzed, or from cheese, which contains no lactose, is absorbed as efficiently as that from milk [22,40,41,105].

Thus, lactose, at the concentrations normally found in milk, seems to have no significant effect on calcium absorption in healthy adults on a normal diet [5]. However, any effect of lactose on passive absorption may be masked by active transport, which is generally sufficient if the dietary intake of calcium is moderate and there is no lack of vitamin D. Lactose may be more important if calcium intake is high, especially in babies and the elderly, in whom solubility is a limiting factor and passive absorption is the predominant route [35]. Lactase deficiency does not prevent the calcium in milk from being well absorbed [36,41,42]. According to the excellent review by Scrimshaw and Murray [43] on lactose intolerance, which is prevalent in most of the world, with the notable exception of people originating from western and central Europe, even alactasic subjects can tolerate 250 g of milk per day and, thus, benefit from its calcium.

Meals have a major effect on the absorption of insoluble calcium supplements like calcium carbonate. Calcium carbonate is better absorbed when given as part of a meal than when it is given without food, particularly in fasting subjects. This has been clearly shown in humans [44] and in pigs [45] and is likely due to the calcium's being dissolved by the gastric juices and to slower gastric emptying.

Dietary Factors Influencing the Excretion of Calcium in Urine

Contrarily to the simultaneous intake of phosphorus, which can be confused with the meal effect (all common foods are rich in phosphorus), and certain constituents that raise the pH (bicarbonate, potassium salts), all the other dietary factors that have an effect at the kidney level increase the urinary loss of calcium generally by reducing tubular reabsorption [46].

Phosphorus may have a direct effect by increasing the reabsorption of calcium in the distal part of the nephron or an indirect effect by stimulating PTH secretion or by enhancing the uptake of absorbed calcium into bone [47]. The simultaneous absorption of calcium and phosphorus increases the uptake of calcium by bone, thereby decreasing its loss in urine [45].

Excess protein generally leads to an increase in the amount of calcium lost in the urine, which may be masked by the opposing effect of excess P (from dietary components rich in both protein and P). This is especially true for proteins with high contents of sulfur-containing amino acids (cysteine, methionine), the breakdown of which releases sulfur oxidized as sulfate, causing moderate acidosis and increasing the excretion of calcium in the urine [48,49]. Sulfate ions also bind calcium, preventing its tubular reabsorption [46,50–52] and even its incorporation into bone [53]. It is therefore not surprising that an excess of protein rich in sulfur amino acids or other sources of sulfate (certain mineral waters) causes more calcium to be lost in the urine than other foods, such as those eaten as part of a vegetarian diet or with bicarbonates [49,54,55].

Chronic metabolic acidosis due to excessive intakes of sulfate and chloride anions leads to higher losses of calcium in

the urine. The alkalosis resulting from ingestion of bicarbonate or potassium citrate has the opposite effect [55].

It has long been known that the renal clearance of calcium is linked to that of sodium. As almost all ingested sodium is excreted in the urine, this effect is particularly sensitive and several groups have developed equations describing it [46,56–60]. According to these equations, every extra two grams of dietary sodium increases urinary calcium excretion by an average of 30 to 40 milligrams.

Clearly, dietary factors affecting the amount of calcium lost in the urine have a major influence on calcium balance and may even be more important than those that influence the intestinal availability of calcium [17]. This is why the inevitable loss of calcium in the urine (accounting for a large part of the maintenance requirement) is greater for Western-type diets that are high in unfavorable factors such as animal protein, sulfates, sodium, coffee, tea and alcohol, than for other diets with lower levels of consumption of these factors.

METHODS FOR MEASURING CALCIUM BIOAVAILABILITY

In vitro Tests

Solubility in a slightly acidic medium is necessary, but not sufficient for bioavailability. The first step in the absorption of certain insoluble calcium supplements, given as tablets, is their disintegration and dissolution in the stomach. The solubility of $CaCO_3$ tablets is investigated using a kinetic test (USP) of dissolution in acetic acid (vinegar) in the US. Other primary tests of absorbability use dialysis, ultrafiltration and various membrane techniques, particularly the isolated intestinal loop, pieces of mucosa or cell layers (caco-2 cells). None of these methods takes into account the whole range of nutritional, physiological and ecological factors that influence absorption, and none provides results directly comparable with those obtained *in vivo* using whole animals.

Classical Balance Studies

This is the only method that provides true, absolute data on absorbability and bioavailability. It also gives mean values, although these are only valid for the period tested, and the test period must be at least one week after starting the diet (but several weeks are often needed). The balance method provides data for apparent (intake—fecal) absorption and net retention (intake—fecal—urinary) and the corresponding coefficients. Isotope dilution studies, using a stable or radioactive isotope of calcium, injected at the start of the evaluation, give the fecal loss of endogenous calcium and, hence, true absorption (intake—exogenous fecal).

Balance studies are slow, labor-intensive and expensive. The validity of the results obtained depends on how accurately the intake and output parameters are estimated. Even under the most rigorous experimental conditions (animals in metabolic cages) the inevitable small errors in assessment of intake (measured by excess) and fecal and urinary losses (measured by defect) always lead to an overestimation of the amounts retained. This overestimation may be very large when retention is low, as is always the case for adults.

Balance studies are essential for estimating the dietary needs of growing animals by the factorial method, but they are of little use for studies on human adults. Even under the most rigorous conditions (several days in a metabolic unit), the measurements are poorly reliable. Consequently, too much of the work published on human adults (normally in negative balance or equilibrium) has indicated that the individuals tested had a positive daily calcium balance as high as 200 to 300 mg calcium, which is most unlikely.

Fortunately, it is not necessary to carry out absolute balance studies or obtain absorption and retention coefficients for the comparison of several dietary sources of calcium. This can be achieved with values given relative to a reference source. This method only provides the bioavailability of calcium for an average diet over the test period for human subjects and cannot be used to compare two sources of calcium. Calcium sources can only be compared if the calcium is given as a single load, a test meal, giving the bioavailability of calcium at that time point only. One method used for human subjects [61,62] involves a preliminary intestinal lavage with isotonic solution followed by the test meal and, then, 12 hours later, a second intestinal lavage to collect the unabsorbed fecal residue. This rather drastic and unphysiological method has been used to show that there is little difference in the bioavailabilities of soluble and poorly soluble calcium salts [63].

Isotope Balance Methods

As in classical balance studies, all feces and urine must be collected over a period of several days, but the balance is calculated only on the tracer isotope in the source of calcium being studied. The intake is known accurately because it is a single dose of radioactive (^{45}Ca, ^{47}Ca) or stable (^{42}Ca, ^{44}Ca, ^{46}Ca or ^{48}Ca) isotope given in a test meal. The absorption and retention coefficients obtained are regarded as being representative of all the calcium in the labeled source.

Unlike the classical balance, the isotope test measures only the instantaneous bioavailability of a single dose taken as part of a meal. There is generally no period of adaptation, and variations over time are not taken into account, even though the coefficient of variation between meals and between days is probably over 10% [64]. The results obtained depend greatly on the experimental protocol, particularly the timing of the operations, such as whether the isotope is given to the fasting subject before, with or after the meal.

One of the main problems with assessments involving isotope tracers (see below) is the labeling technique itself. Ideally, an intrinsic marker should be used; for example, calcium in

milk can be labeled by giving the cow several injections of ^{45}Ca, whereas plant calcium can be labeled by adding the isotope to the fertilizer. Most labeling is extrinsic, however: this means that the food to be studied is mixed with the isotope, $^{45}CaCl_2$, for example. This assumes that there is a perfect exchange between the calcium in the foodstuff and the added isotope. Most dairy products seem to come rapidly to equilibrium [65], as do many other foodstuffs [66], but this is not true for certain plant products that contain insoluble calcium salts such as phytates and oxalates [67]. The bioavailability of calcium in these foods may therefore be considerably overestimated.

Urinary Excretion of an Oral Calcium Load

This is one of the methods most frequently used to compare sources of calcium in human studies. Unlike some animals (e.g., rats and pigs), which lose little or less calcium via the urine, humans excrete large amounts of calcium in urine. The increase in the amount of calcium lost after a calcium load is given to a fasting subject (about 500 mg Ca) may be thought of as reflecting the effectiveness of calcium absorption. However, the results reflect instant absorbability and also depend on several dietary factors that affect the loss of calcium in urine, by reducing it (phosphorus) or increasing it (sodium, high-sulfur protein, sulfate, certain carbohydrates).

This test is simple and fast. A urinary response can be obtained three to four hours after ingesting the test meal, and the urinary calcium data (relative to urinary creatinine) can be used to compare different sources of calcium [68–70]. Variations on this test use test meals labeled with stable isotopes.

Measuring Isotopes Labeled in the Blood, Urine or Bone

This may involve a single label for the rapid comparison (two to four hours after a single oral load) of labeled sources of calcium by measuring (sometimes with kinetic studies) radioactivity or stable isotope enrichment in the blood, urine or bone (used particularly for animals). An estimate of true relative absorption may also be obtained by measuring the area under the plasma isotope concentration curve. The direct measurement of the radioactivity taken up by a representative bone is possible using the ^{47}Ca isotope (a gamma ray emitter).

The most commonly used method at present is a double-label method in which a test meal labeled with one Ca isotope is ingested and a second Ca isotope is injected intravenously. The behavior of this second isotope reflects, in principle, 100% absorption. The isotope concentrations are measured later two to four hours in the blood, 24–36 hours in the urine). The ratio of the two isotopes (ingested and injected) is assumed to be equal to the fractional absorption (between 0 and 1) of the test calcium. Several sources of calcium can be compared rapidly and absolute true absorbability determined over several days without collecting feces. In addition, as these assays are relatively short in duration, they can be repeated on the same subjects after allowing for a "decontamination" interval.

This double radioisotope labeling technique has been widely used in animals and in humans [71] A rapid method in which one radioisotope of calcium is injected, followed by a second injection of the same isotope 2 hours later has been devised by Chanard *et al.*, [72] and used routinely by Wynckel *et al.* [73]. Stable isotopes are now used in double-label studies in humans [74]. The validity of several variations of this method, differing in the type of blood sample or urine sample used and in the method of calculation, has recently been analyzed [75].

Several accurate mass spectrometry methods for measuring the enrichment of stable isotopes of calcium are now available [76]. The validity of bioavailability assessments based on these techniques depends, however, on several factors, including the quality of labeling of the test load, the representative nature of the samples and variations in the physiological and nutritional status of the experimental subjects.

Long-Term Evaluation of Bone Mineralization

Measuring bone parameters after prolonged treatment (several weeks for growing animals) is undoubtedly the most reliable way of estimating the long-term effects of qualitatively and quantitatively different calcium intakes. The mineral content, mineral density, breaking strength and morphometric parameters of a representative bone can be measured for experimental animals once they have been killed. The best methods currently available for measuring bone mass in several parts of the human skeleton are double X-ray absorptiometry (DEXA) or quantitative computed tomography (QCT) for lumbar vertebrae.

These bone criteria are generally sensitive enough for comparing sources of calcium, provided that the subjects are young, and reactive, with large calcium requirements and that they are assayed over a sufficiently long period. A single calcium intake concentration can be used for several sources, but it is better to use several intake concentrations for each source. This provides bone responses that vary with the intake. The slope-ratio of the curves for each source gives the bioavailability. This method gives very good results because it eliminates the large effect of small changes in calcium intake.

It is easier to interpret these data if the basal diet is low in calcium, because then almost all the calcium ingested comes from the test source, provided that all the other dietary factors affecting calcium absorption, such as proteins, phosphorus, phytates and sodium, remain the same.

Measuring of Biological Markers in the Blood or Urine

The concentration of PTH in the plasma falls when there is a small transient increase in plasma calcium concentration (or

in Ca^{++}) due to the intestinal absorption of an oral calcium load. This transient decrease is, however, proportional to the efficiency of absorption. This test is easy to perform in short-term comparative studies on human subjects, but it does not take into account further urinary loss of calcium, hence its retention by bone.

Some factors in the blood or urine vary with the degree of bone accretion or resorption. They can therefore be used in comparative tests to measure the effect of various amounts of absorbed calcium. The loss of hydroxyproline in urine is an indicator of bone resorption. It is now used in complement with or has been replaced by assays of more specific bone markers, collagen "cross-links", pyridinoline or, better still, deoxypyridinoline.

SELECTION OF ANIMAL MODELS

In vitro tests can be used to detect factors likely to alter the intestinal absorbability of calcium, but they are not really of use for quantifying bioavailability and cannot replace *in vivo* trials on animals. Experiments in humans are, of course, ultimately required, but it is still necessary, for many reasons, to carry out animal studies.

Selecting a Species

The main species used are rats, pigs, guinea pigs and primates. The dietary behavior of the animal must be taken into account, including the type of diet and frequency of meals, for example. Pigs are omnivores that eat rapidly two or three meals per day. This similarity to human behavior makes them an ideal model. Rats and guinea pigs eat grain and are continuous nibblers or gnawers without well defined meals.

The physiological characteristics of the rat also make it an unsuitable model. Its intestine presents high levels of phytase activity enabling it to hydrolyze phytates in food and to absorb calcium down as far as the large intestine. Neither pigs nor humans are able to do this, at least not to the same extent. The main problem with guinea pigs, rabbits and, to a lesser extent rats, is that they are coprophagous, a circumstance which makes interpretation of true absorption results complicated.

Rats are poorer animal models than pigs for studies on bone metabolism because their skeletons are continuously growing and never reach a bone remodeling stage paralleling that of human adults. In pigs, closure of epiphyseal cartilage occurs at the age of two to four years [77]. There is, however, no evidence that this difference, which may be important when studying factors affecting osteoporosis [78], has any effect on the absorptive capacity of the intestine.

Pigs and rats lose very little calcium in the urine, whereas humans and guinea pigs have very high urinary calcium levels. This factor limits the suitability of pigs for use in studies on the factors that may influence urinary calcium levels in humans. The lack of renal excretion of the excess absorbed calcium is probably offset in pigs by greater elimination via the endogenous fecal route.

Interest and Advantages of Animal Experiments

There is no doubt that it is preferable to carry out experiments on animals than on humans for ethical, material and financial reasons. Clearly it is much more feasible to work with young growing animals, whose calcium metabolism is very active, than to attempt such studies on children. Such experiments are important because the coefficients of calcium absorption and retention often depend more on the physiological condition of the subject than on the nature of the calcium ingested. Comparative studies on the bioavailability of several sources of calcium must therefore make use of subjects that are physiologically capable of retaining the ingested calcium.

Several technical manipulations are possible, such as the insertion of intestinal cannulae or catheters for repeated blood sampling. Radioisotopes are less expensive to use than stable isotopes, and they are also easier to measure accurately. Long-term trials involving extended periods in metabolic cages can be used to accurately measure ingested and excreted amounts; such trials are far from easy in humans. It is also possible to take organ samples from animals killed at a specific stage, which is of particular value for representative bone samples for a range of chemical, biological, morphometric and histological tests and for mechanical tests of breaking strength.

The power of the statistical tests that can be used is much greater with animal models. It is easy to set up very uniform groups with most of the animal species used (except, perhaps, primates), with very little variation between individuals and parameters like breed, strain, gender, age, weight, physiological state and dietary history all the same. Dietary components may be altered as required and the amounts consumed and excreted are accurately known.

It is thus possible to detect small but statistically significant differences between groups of ten individuals for animals, whereas dozens or even hundreds of individuals per group would be required if the experiments were done on humans. For example, we know that the average urinary loss of calcium in a human adult is 150±50 mg Ca/day (coefficient of variation=30%). Therefore, an increase of 15 mg per day in response to a dietary factor (e.g., sulfate) can only be statistically significant if the trial includes at least 100 subjects per group in a long term cross-over trial or many more subjects per group if it is a short-term trial with two groups. In contrast, this type of small effect is readily demonstrated in animals and has a very considerable long-term physiological consequence. Among other examples, the recent experiment done by Couzy *et al.* [74] shows the limits of human experiments. They concluded that sulfates in mineral water had no effect on urinary calcium loss, relative to milk, because the observed 14% increase was at the limit of statistical significance. In fact, because of the large

variation between individuals that is inevitable in this type of study, such a difference between two groups containing only nine adult subjects cannot be significant. However, the increases in urinary sulfate (+35%) and magnesium (+18%) were significant at the 5% level.

Only animal experiments can be used to show the statistical significance of small changes, and their demonstration in animals indicates that they may also exist in humans.

COMPARATIVE BIOAVAILABILITY OF CALCIUM IN FOODS: REVIEW

Comparison of Sources of Calcium

Human Studies. Many trials have been carried out over the past 15 years to compare calcium in milk with several other sources of calcium, such as salts, mineral waters and plant products. Almost 20 of the studies on bioavailability were carried out on men or women, using a variety of methods (true or apparent absorption, urinary calcium). None of the studies showed that the calcium in milk was more efficiently used than any calcium salt. Carbonate, gluconolactate, citromalate (CCM), chloride, lactate, acetate and citrate were tested [40,44,62,79–88]. The calcium from mineral water, bicarbonate or sulfate, was not found to be any better for absorption [73,74,89,90]. The findings were similar for several milk derivatives (yogurts, cheeses, chocolate milk, acidified milk) [40,70,90,91]. The calcium in milk and dairy products is much better absorbed than the calcium in spinach or watercress, as these plants have high oxalate contents [64,84,92–96]. Studies in humans, comparing the absorption of calcium from milk with that of CCM, suggest that calcium availability from CCM is higher [84,87], even than that from calcium carbonate [33,44,84,87]. A study carried out on women showed that the fractional absorption of calcium from cabbage was better than that of calcium from milk [98].

Studies on Rats and Pigs. There have been about 15 studies performed on rats over the past 15 years. They show a similar pattern, but many also include measurements of bone retention of labeled calcium [27,39,65,99,101–103] and tested a wider spectrum of minerals and milk products as sources of calcium that would be possible in studies on humans. Thus, studies in rats show that the calcium in whey is as efficiently absorbed and utilized for bone mineralization as that bound to casein [104,105] and that there is little difference between dairy products in general (milk, acidified milk, yogurt, skim milk, cream cheese, hard cheeses) [27,65,100,106]. Two studies in rats found differences between the "calcium value" of yogurt and milk [102,107], but their findings are contradictory. Studies in humans have shown that calcium absorption from these two sources is similar [40,41,91].

Long term studies in growing pigs [108,109] or ovariectomized mini-pigs [110] have provided no evidence that calcium is better absorbed from milk and milk products (casein phosphopeptides, skim milk or yogurt) than from mineral salts (CCM, $CaCO_3$). However, bone mineralization (evaluated by breaking strength) is better in animals fed yogurt as a calcium source than in those that obtain their calcium mainly from minerals ($CaHPO_4 + CaCO_3$) [109].

As in humans, most trials in rats have found no difference between the use of Ca from yogurt and that from other milk or mineral sources [27,40,41,65,91,100]. However, adding yogurt to the diet improves the fractional absorption of calcium [111]. Calcium in cheese is as efficiently used as the calcium in milk or carbonate [40,65,70,91,106]. A study on growing rats by Dupuis *et al.* [27] found that calcium is initially better retained from milk products than from calcium carbonate, but that this difference is later lost. Calcium from plants (apart from cabbage and some other crucifers), particularly that from cereals, is generally less well absorbed than the calcium from milk [112–114]. Phytates (present in large amounts in wheat bran and in soybean-based products) reduce the absorption of calcium from calcium carbonate [115] and from milk [116]. A study on rats using goat milk products found that the calcium from goat's cheese is less well retained than that from milk [65]. Only one study in rats found that increasing the dietary calcium intake with calcium sulfate leads to an increase, in four weeks, in bone mineral content. The same calcium intake from milk provides similar (ash as % dry matter) or higher levels of mineralization (Ca as a % of bone dry matter) than that provided by calcium sulfate [113]. The bioavailability of calcium from milk was estimated to be 113% that of calcium from calcium sulfate in this study.

To summarize. The mean apparent calcium absorption (% intake) from all collected data concerning calcium salts from human studies varied from 23% to 37%, excluding phosphates, because of the too large range of variation and the paucity of data. The following averages have been calculated from 3 to 8 references (citrate, citromalate, chloride, lactate, gluconate or a mixture of lactate and gluconate) to 12 to 14 references (carbonate, milk): carbonate, from 26.4 (fasting) to 29 (meal), citromalate from 32 (fasting) to 37 (meal), citrate 23.5 (fasting), lactate+gluconate 24.5 (fasting), chloride 30.6 (fasting), milk 32.4, cheese 32.8, mineral waters 32.3, oxalate-rich products (calcium oxalate, spinach, watercress) 13.2. These values are to be considered with care because they result from trials that compare different diets, ages and many other parameters. Furthermore, as suggested above, some calcium sources have been well investigated and some not.

Calcium Absorption *versus* Bone Retention

Intestinal absorption does not necessarily reflect the bioavailability to the whole organism because calcium must be retained and used in bone formation and mineralization. Phosphorus must also be present for the production of hydroxyapatite (a complex tricalcium phosphate). The dissociation of

calcium intake from that of phosphorus (if, for example, the calcium source is not ingested with the meal and/or this source contains no P), may restrict bone mineralization. This has been known for some time and was recently confirmed in growing pigs, which are extremely sensitive to dietary mineral supplies [45,117].

Three sources of calcium, calcium carbonate, CCM and milk, have been extensively studied. They all ensure the efficient absorption of calcium and also, over a long term (one to four years), that calcium is retained and used for bone mineralization. This was reported by Prince *et al.* [118] in a study on menopausal women, in whom calcium supplements, given as $CaCO_3$ tablets or as milk, reduce the bone loss measured over a two year period. Similarly, Smith *et al.* [119] studied 169 women aged from 35 to 65 years who were given calcium carbonate supplements (or a placebo) for four years. The calcium carbonate supplements reduced bone loss around menopause (bone mineralization study) at 12 sites. A group of adolescents was given a calcium supplement (one g/day, from 900 mL milk or calcium carbonate tablets), and it was found that bone density, determined 10, 18 and 24 months later, was higher in those given calcium than in those given the placebo and that the milk and carbonate sources were equally effective [120]. Recently [121] it was reported that calcium-enriched foods significantly increased bone mass accrual in prepubertal girls, with a preferential effect in the appendicular skeleton and greater benefit at lower spontaneous calcium intake. Lastly, postmenopausal bone loss was reduced at the main sites of spongy bone (but not of cortical bone) by supplementing calcium intake with calcium carbonate or CCM in a two year study by Dawson-Hughes *et al.* [122]. CCM was found to be the most effective. Other salts have been used in long-term studies (tricalcium phosphate, glucono-lactate plus calcium carbonate) and shown to reduce bone loss or the incidence of hip fracture [123,124].

Such longitudinal clinical studies have yet to be done using mineral water as the source of calcium. Hence, there is, as yet, no evidence showing that calcium from mineral water is similarly effective. While several human studies indicate that calcium from these sources is as well absorbed as that from milk or calcium carbonate, the effect of prolonged mineral water consumption on bone mineralization is not yet clear. Only one study, that of Cepollaro *et al.*, [125] reported a positive effect of consuming a high-calcium bicarbonate water on the bone density of 45 menopausal women, after 13 months of this form of supplementation. The control group (who drank a low-calcium water) was given no calcium supplement (calcium intake: supplemented, 1500 mg/day; non-supplemented, 949 mg/day). Apart from this trial, there have only been very short-term studies (a few days) for high-calcium mineral waters, and such studies are too short to test for any bone effect [74]. Careful interpretation is therefore required; the efficient absorption of calcium from these high-sulfate, high-bicarbonate waters, similar to that of calcium from milk or carbonate, does not necessarily show that this calcium is as well retained by bone. A recent preliminary report [126] showed that giving a calcium supplement in the form of calcium-rich water to postmenopausal women for two months reduced bone resorption (determined by the excretion of markers of bone resorption), but that the effect was much less marked with high-sulfate water than with high-bicarbonate water. It is well known that the urinary loss of calcium is lower with alkalogenic diets, rich in vegetables and fruits or bicarbonates [54,127]. The problem of urinary loss of calcium with these calcium sulfate sources remains to be determined over longer periods.

Our recent studies in growing pigs have shown greater bone mineralization (measured as ash, density and breaking strength of various bones) in pigs fed a "milk" diet (70% of the calcium intake as powdered skim milk) than in pigs fed a "sulfate" diet (50% of total Ca intake as $CaSO_4$ and 33% as $CaCO_3$) or a "carbonate" diet (80% of intake). The sulfate and carbonate diets gave similar levels of mineralization. All the diets had the same energy, protein and calcium contents (Pointillart and Guéguen, unpublished results).

Casein Phosphopeptides, Proteins and Calcium Availability

The positive effects of milk casein phosphopeptides (CPP) on the absorption of calcium have been shown mainly with *in vitro* studies of calcium transfer (ligated intestinal loops or everted sacs) in rats [128–133] and in a few *in vivo* studies in rats in which calcium absorption and bone retention were measured [134–137]. The CPP were compared to soybean protein extracts, egg white [135], wheat gluten or gelatin [129] or fibrin [131]. A study on isolated chicken intestinal loops also showed that CPP increased calcium transfer [14]. A diet in which 50% of calcium and about 33% of P are provided by CPP has no effect on calcium absorption or bone retention in pigs [108]. Feeding of casein, a potential substrate to the release of CPP, to growing miniature pigs improved femur mineralization as compared to whey protein. This observation was true when vitamin D deficient diets were given but not when adequate vitamin D supply was provided [137].

Studies on unweaned babies show that those fed soybean-based formula have 25% less bone mineralization (from densitometry measurements) than those fed milk-based formula [138,139]. Conversely, *in vivo* studies on ovariectomized rats showed that these animals lost less bone if fed a diet containing soybean protein extract than if fed a milk-based diet [140, 141]. The authors interpreted this as being due to the phytoestrogens in soybean. It is difficult to extrapolate these results to humans, given that Tsuchita *et al.* [142] clearly showed less bone loss following ovariectomy in rats fed CPP than in rats given Ca and P as pure minerals.

Partridge [143] showed greater calcium absorption in very young pigs fed milk than in those fed an isocalcium diet containing soybean meal. Similar results were obtained in pigs

by Matsui *et al.* [144]. The opposite pattern is later observed (soyabean>milk) in pigs aged four months, and there is no difference in older animals (soya=milk).

The positive estrogen-mimetic effect on bone has only been observed in ovariectomized rats, and soybean products have a high phytate content which may reduce calcium absorption, as has been clearly demonstrated, including in women [116,145]. Lastly, several *in vivo* studies have shown that calcium in diets with various soyabean and CPP contents is similarly absorbed [rat: 104,146,147; pig:108]. A clinical study on unweaned babies up to six months old compared bone density at various stages of development, and found no difference between mother's milk and formulas based on soybean or on cow's milk [148]. In contrast, the amount of animal protein consumed by women was found to be strongly correlated with the incidence of hip fracture in a retrospective epidemiological study carried out by Abelow *et al.* [149].

It has been shown in many studies that the greater the amount of dietary protein, the higher the urinary calcium level, regardless of whether the protein is casein or soybean protein [in rats: 147; in man: reviewed by Abelow *et al.*, 149]. Thus, high levels of protein consumption lead to a negative calcium balance. Reducing the milk protein content of the diet reduces urinary calcium loss in man [150]. Calcium supplementation in the form of milk increases the amount of sulfate in urine because milk has a high content of sulfur-containing amino acids [80], and some studies have implicated these amino acids in the hypercalciuria and negative calcium balance associated with diets containing too much animal protein [51,55,147,151–153]. A horizontal study carried out in China on women who had consumed a variety of diets (with and without animal protein, plus or minus milk) indicated a greater correlation between urinary calcium and the consumption of animal proteins not derived from milk [154]. However, things are not that simple. A study performed by Allen *et al.* [155] on humans with controlled diets and for whom the dietary protein was tripled from 12 g N/day to 36 g N/day by adding soya extract clearly showed an increased urinary calcium loss (1.5-fold) which changed the calcium balance from −37 mg/day for 12 g N/day to −137 mg/day on 36 g N/day, despite high calcium intakes (1400 mg/day) and the similar absorptions. This problem of the effect of excess protein on bone has been recently discussed [55].

In Conclusion. While the proteins in milk or milk products may have beneficial effects on bone mineralization, this is not always so. In contrast, the positive effects of soya on calcium retention have only been demonstrated in one rather special system, ovariectomized rats, while it has been clearly shown that the phytates in soya can reduce calcium absorption in humans. Both milk and CPP have a favorable effect on calcium absorption. The high phosphorus content of milk may offset the hypercalciuria induced by protein [156], although the intakes of both calcium and phosphorus provided by the milk help promote bone mineralization.

The hypercalciuric effects of high-protein diets, particularly those containing animal proteins, are well known. However, human studies on the nature of these proteins, plant/animal, milk/non-milk proteins, and their long term influence on calcium balance or bone metabolism are still necessary before we can come to any conclusion about whether plant proteins are advantageous or not. Thus, particularly strict vegetarian diets that contain no milk products may present risks to bone mineralization [157] because they do not provide an adequate calcium intake, without recourse to supplements provided by mineral calcium tablets [114].

Lactose, Lactase and Calcium Bioavailability

In Rats. Lactose is reputed to stimulate calcium absorption and most of the experimental evidence for this has been obtained in rats [21,25,26,29,39,158]. These *in vivo* studies provide direct evidence that it acts on the intestines and on bone. There is also indirect, *in vitro*, evidence obtained from studies on isolated intestinal loops [31,159] in which lactose was compared to another sugar or the absence of lactose [159]. Other studies on isolated gut loops *in situ* have, however, shown that 30% lactose can reduce the absorption of calcium chloride [15]. There is also other indirect experimental evidence. For example, *in vivo* studies have compared the calcium absorbed from normal milk and from milk in which the lactose had been hydrolyzed [39]. Others have shown that lactose, unlike sucrose, reduces the effects of a lack of vitamin D on bone [5]. Lastly, adding lactose to cheddar cheese was found to give better calcium absorption than with cheese alone [106].

In Humans. The effect of lactose is perhaps less clear cut in man because it is complicated by the problem of lactose intolerance and thus of a lactase deficiency [see review by Scrimshaw and Murray: 43]. Griessen *et al.* [36] found that lactose increased the fractional absorption of calcium in lactase deficient (LD) patients, but most studies have shown a reverse effect [38,160–162] or no effect [37]. A group of five studies showed that the presence of lactose, or its addition, stimulated calcium absorption in lactose-tolerant subjects [35,38,161,163,164], but three other studies demonstrated no effect [37,165,166].

There is no real proof that hypolactasic patients absorb calcium less well than others. At least one study [37] found that the absorption of calcium from a standard diet by lactase-deficient patients was better than that of lactose-tolerant patients, another found that it was poorer [160], while still others have reported that the basal absorptions were similar [36,38,41,111], even when there was milk or yogurt in the diet [41]. Yogurt can increase calcium absorption in both LD and non-LD subjects [111] compared to a $CaCl_2$ solution.

Normal mother's milk results in better absorption of calcium by unweaned babies than when the lactose is removed, and adding lactase to mother's milk can increase calcium absorption [163]. Lactose seems to have an even greater effect

on calcium absorption when absorption is basically poor [35,164].

A recent clinical study [167] on children about 10 years old found no difference between the bone densities of lactose-intolerant and paired (height and weight) controls, but they did find that there was a strong correlation ($r=0.9$) between bone density and calcium intake in the lactose-intolerant children. Several epidemiological studies have also shown that lactose-intolerant subjects consume less calcium (from dairy products), which may predispose them to osteoporosis [168,169]. This link is not always found, as indicated by the lack of a difference between the bone densities of female twins, one of whom was hypolactasic and the other lactose tolerant (in response to an oral load) [170]. The answer may lie in the total amount of Ca consumed, i.e. from dairy and non-dairy foods.

In conclusion. Several studies have shown that lactose has a positive effect on calcium utilization, but there is some uncertainty, at least in LD people in whom lactose can reduce absorption. It is possible that this effect is only temporary, as suggested by certain studies on the changes in calcium absorption after a meal [38]. The literature contains many contradictions concerning the reduced absorption in LD subjects. It is quite probable that the non-consumption of dairy products by these subjects tends to reduce their calcium intake, but that could be offset by more efficient absorption, provided that they still have the capacity to adapt; it is far from clear that the elderly have such a capacity.

Influence of Dairy Product Consumption on Bone Density

All of the 14 clinical or epidemiological studies published over the past decade [118,171–181], except one [182], have shown that the consumption of dairy products in childhood and adolescence has a positive effect on bone mineralization later in life, as assessed by bone density measured at several sites in adults. They therefore confirm the classic findings of Matkovic *et al.* [183]. This effect on subsequent bone density is reduced or lost when milk is consumed between the ages of 20 and 30 [172,173,179]. Several studies have found that a dairy product supplement increased bone density in adolescents [174,177] or reduced bone loss in post-menopausal women [118]. Lastly, osteoporotic women were found to have consumed less dairy product than healthy controls when they were children and adolescents [172,175]. A recent review [184] done on children found that consumption of extra calcium increased their bone density 1% to 5% or even 10% when the source of calcium was dairy products. This was recently confirmed with a double-bind, placebo-controlled trial in prepubertal girls [121]. The question remains on whether such effects persist after six to 36 months of intervention.

Only one study, that by Prince *et al.* [118], has compared the effects of calcium supplements, given as 1-g/day mineral tablets or dairy products, for two years on women at least 10 years after menopause. Both types of supplements have similar effects: they reduce bone loss from several sites in the hip, but not from the lumbar vertebrae. The findings of a number of recent meta-analyses of data from horizontal studies looking for a link between calcium intake and bone loss have arrived at contradictory conclusions. However, prospective studies on the effects of calcium supplements have generally shown that it has a positive impact on bone loss [185,186]. According to Nordin [187], some contradictory conclusions could be due to errors in the dietary data. Nordin analyzed 19 trials, three using dairy products. This analysis clearly showed that calcium supplementation reduced bone loss from 1.26% per year in controls to 0.12% in those receiving calcium supplements ($p<0.005$). These are data for the bone densities of 1300 postmenopausal women, measured at 11 bone sites, including cortical and/or trabecular bone. The difference between the annual percent bone loss between supplemented and unsupplemented women varied from +0.28 (spine) to +4.1 (femoral diaphysis). Lastly, Lyritis *et al.* [188] found a correlation between the consumption of dairy products by young adult humans and their bone density.

We can therefore say that a greater calcium intake, particularly of milk products, during the period of peak bone formation has a positive effect on bone density of adults and undoubtedly reduces the risk of osteoporosis. But only intervention studies have shown that calcium supplementation has a beneficial effect on bone loss, while the results of horizontal epidemiological studies are more controversial.

PECULIARITIES AND ADVANTAGES OF THE CALCIUM IN MILK AND DAIRY PRODUCTS

It is well worth remembering that milk and milk products are by far the main source of calcium in our diet [1]. Cow's milk contains an average of 1.20 g calcium per liter, 20% of which is bound to casein as an insoluble organic colloid and the remaining 80% in mineral form (45% in the tricalcium phosphate of the phospho-caseinate, which is also insoluble and colloidal, and 35% soluble, including 12% as ionized calcium) [189]. The organic or mineral calcium bound to casein is readily released during digestion, and there is general agreement that its potential bioavailability is high. Most solubility studies use milk calcium as a reference standard. The calcium in spinach, which is present as an insoluble oxalate, is taken as the extreme example of poor bioavailability. However, except for newborns fed on mother's milk (calves drinking cow's milk) which can absorb almost all the ingested calcium, the percent of milk calcium absorbed seldom exceeds 40% under normal dietary conditions.

The calcium in cheeses is readily available, despite the fact that cheese often contains large amounts of saturated long chain

fatty acids and no lactose [22]. Tests on rats fed cheddar cheese labeled with ^{47}Ca showed that the calcium was as well absorbed as was that from milk and that absorption was not influenced by the maturation time [106].

There is therefore no difference in the availability of calcium from milk and most of the best mineral or organic sources of calcium which are often used as medicines or dietary supplements and whose coefficient of absorption is about 30% to 40%. Only a few organic forms, like citrate-malate, can provide slightly better calcium availability [2].

Nevertheless, the calcium in milk differs in several interesting features from the calcium in other foodstuffs or supplements. These can be important when it is necessary to ensure high absorption of calcium under unfavorable physiological conditions [35]. Because it is bound to peptides and proteins, milk calcium is more likely to remain in solution when the pH is unfavorable, such as in achlorhydria. Milk calcium may be absorbed in the absence of vitamin D, under the influence of lactose in the distal small intestine via the paracellular route. Thus milk can provide calcium with "ensured absorbability" which is generally insensitive to external factors, except for inhibitors, such as oxalic acid. Dairy products do not contain anything likely to inhibit the intestinal absorption of calcium, like phytates, oxalates, uronic acids or the polyphenols of certain plant foods. The hypercalciuric effect of sulfates from milk proteins is offset by the hypocalciuric effect of phosphorus [156]. The endogenous sulfates produced by the breakdown of sulfur-containing amino acids produces a SO_4/Ca ratio of 0.6, while this ratio is 2.6 in some high-sulfate, high-calcium mineral waters.

Lastly, it should be remembered that milk and dairy products are not only excellent sources of calcium, but also provide an almost complete diet whose consumption provides a "meal effect" [17]. This fosters the absorption of calcium and provides a simultaneous intake of phosphorus that is essential for bone deposition. These advantages cannot be provided by any other source of calcium, such as calcium supplements or Ca-rich waters.

As milk provides calcium with "protected absorbability," "prolonged absorption" and "extended bone deposition," milk is the most suitable dietary constituent that meets the high calcium intake required by postmenopausal women and the elderly. This is especially important because, according to some workers [176], and for still unknown reasons, the inhibition of bone remodeling that generally occurs in response to a high calcium intake is less marked when calcium is supplied by milk products. Further studies are now needed to identify a possible specific effect of milk products on bone, although this beneficial effect could be simply due to different rates of calcium absorption, with slower gastric emptying and a prolonged passive diffusion that ensures an extended supply of calcium to the bone.

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The Bioavailability of Dietary Calcium

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This update focuses on the bioavailability of dietary calcium for humans. Fundamentals of calcium metabolism, intestinal absorption, urinary excretion and balance are recalled. Dietary factors, especially lactose and other milk components, influencing calcium bioavailability at intestinal and renal levels are reviewed. A critical examination of all the methods used for evaluating calcium bioavailability is made. This includes *in vitro* assays, classical and isotopic balances, urinary excretion, isotope labeling in the urine, plasma and bones, long-term evaluation of bone mineralization and the use of biological bone markers. Importance and advantages of animal models are discussed. The state of the art in the comparative bioavailability of calcium in foods is detailed including a comparison of sources of calcium (dairy products and calcium salts) in human studies and in some animal studies, casein phosphopeptides, proteins, lactose and lactase and their relation with calcium bioavailability (in humans and rats). An update on the consumption of dairy products and bone mass is presented. Emphasis on peculiarities and advantages of calcium in milk and dairy products is given.

Key teaching points:

- Milk provides large amounts of calcium and phosphorus and components such as lactose and casein phosphopeptides which may enhance calcium absorption and mineral retention.
- Using a variety of methods, no one has shown that the calcium in milk is more efficiently absorbed that most calcium salts.
- Intestinal absorption does not necessarily reflect the bioavailability of calcium to the whole organism because calcium must be retained and used in bone formation and mineralization.
- Three sources of calcium, milk, calcium carbonate and calcium citromalate, have been extensively studied. They all ensure the efficient absorption of calcium and also ensure, over the long term, that calcium is retained and used for bone mineralization.
- There is, as yet, no evidence showing that the calcium from mineral water is as effective.
- Many direct or indirect methods may be used to evaluate calcium bioavailability. The values obtained depend on the method; thus, conclusions or comparisons must be drawn with care.

INTRODUCTION

Both scientists and the general public are becoming increasingly aware of the importance of dietary calcium. This is largely due to the many research studies that have demonstrated links between dietary calcium intake and diseases such as osteoporosis, arterial hypertension and colon cancer. These diseases have many causes, but the scientific community now recognizes that dietary calcium helps prevent them.

The average recommended dietary allowance (RDA) or adequate intake (AI) of calcium is about 900 mg per day (800 to 1000 mg, depending on the country) for adults, rising to 1200 mg/day for adolescents and the elderly. These RDAs are safety levels designed to provide adults with maximum protection against a negative calcium balance and, hence, against bone loss. But they are also set so as to ensure that adolescents produce the maximum amount of bone that is genetically possible and, hence, remain above the fracture threshold when they become older.

A recent review of calcium consumption in France [1] determined the percentage of each sector of the population that consumed less than two thirds of the RDA, the critical threshold for defining groups at risk. These groups included about 20% to 25% of men aged 18 to 65, 30% of women aged 18 to 50, 50% of adolescent girls and men aged over 65, and 75% of women over the age of 55. Elderly women living in institutions had particularly low calcium intakes.

About 70% of dietary calcium comes from milk and dairy products, mainly cheese in adults. Only a few green vegetables and dried fruits are good sources of calcium (16% of

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intake), and drinking water, including mineral water, provides 6% to 7%.

There is no doubt that milk provides large amounts of calcium. While there is also no question of the nutritional effectiveness of the calcium provided by milk, there is still some debate as to whether this source of calcium is biologically better than other sources, such as calcium salts, certain vegetables or mineral waters. We have therefore included recent publications in which the calcium provided by dairy products is compared to calcium from these other sources. While our coverage is more extensive than that of many other reviews, there has been so much work published on this topic that we cannot claim to have cited all data. For general aspects of calcium metabolism and factors influencing bioavailability, we have drawn extensively on our earlier reviews of calcium availability [2,3] and complementary data may be found in other reviews [4–6].

The review focuses particularity on the bioavailability of calcium from milk and dairy products.

DEFINITIONS AND WAYS OF EXPRESSING BIOAVAILABILITY

Absorbability, or the availability of calcium for absorption by the intestines, is often used as a synonym for bioavailability. It is, however, no more than the first step towards bioavailability. Calcium must be soluble in the acid medium of the stomach before it can be absorbed. Good solubility in water is an advantage but is not absolutely necessary. The intestinal absorption values measured in humans and animals are not always equivalent to, and are generally lower than, calcium absorbability. The potential absorbability of calcium depends on the food, whereas absorption depends also on the absorptive capacity of the intestines, which is affected by physiological factors such as calcium reserves, hormonal regulation or previous dietary calcium supply. The potential absorbability is thus the absorption under the most favorable physiological conditions.

Bioavailability depends on absorbability and the incorporation of absorbed calcium into bone. Hence, it also depends on the urinary excretion and fecal loss of endogenous calcium. As for intestinal absorption, physiological factors, particularly hormones, play a major role in the incorporation of calcium into bone. However, certain types of food increase the likelihood that absorbed calcium will be incorporated into bone, whereas others result in calcium being mainly excreted in the urine. The effects of small changes in the diet on the net calcium balance have been emphasized by several studies. Thus, certain anions, such as sulfate and chloride, organic ligands (chelators) and excess protein or sodium all increase the loss of calcium in the urine and, thus, hinder its incorporation into bone. Conversely, the incorporation of absorbed calcium into bone is stimulated by phosphorus, but excess phosphorus may also cause undesirable ectopic calcification (outside of the bone). The bioavailability of calcium may therefore be defined as the fraction of dietary calcium that is potentially absorbable by the intestine and can be used for physiological functions, particularly bone mineralization, or to limit bone loss.

Absorbability and bioavailability may be absolute or relative. Unless defining dietary needs by the factorial method, relative values are sufficient for determining the fraction absorbed in comparison with different sources of calcium. The values are then expressed relative to a reference source.

The values measured may be mean values or discrete values. Mean values are recorded for a whole diet or a single source of calcium studied over a period of weeks or months after adaptation. Discrete values are for a single meal or a single oral calcium load. They correspond less to normal dietary conditions than mean values and do not take into account the large variations that occur over time.

FUNDAMENTALS OF CALCIUM METABOLISM

Intestinal Absorption

Calcium must be in a soluble form, generally ionized (Ca^{++}), at least in the upper small intestine or bound to a soluble organic molecule before it can cross the wall of the intestine. Absorption is the result of two processes, active transport across cells, mainly in the duodenum and the upper jejunum, and passive diffusion, which occurs throughout the small intestine, but mainly in the ileum [7] and very little in the large intestine [8].

Active Transport. The active transport system for calcium is saturable and regulated by dietary intake and the needs of the body. It involves three stages: entry across the brush border of the enterocyte, diffusion across the cytoplasm and secretion across the basolateral membrane into the extracellular liquid [9,10].

Calcium enters the cell via a positive electrochemical gradient because the calcium concentration in the cytoplasm is very low. It crosses the membrane via calcium channels and via membrane-binding transport proteins (calmodulin and membrane calcium-binding proteins). It may be stored transiently in organelles like the Golgi apparatus, endoplasmic reticulum (ER) or mitochondria, but then crosses the cytoplasm attached to a calcium binding protein (CaBP or calbindin-D 9K), which is the rate limiting factor in active calcium transport. Calcium may travel bound to the protein if the CaBP remains in the cytoplasm or via membrane-bound vesicles if the CaBP is incorporated into the lysosomes [9]. It is extruded from the cell against an electrochemical gradient by two routes. A small fraction leaves by exchanging 3 Na^+ for 2 Ca^{++}, but most leaves via a calcium pump, a Ca-ATPase activated by calcium, CaBP and calmodulin.

Vitamin D influences several steps in this active transport. The active metabolite is 1,25 dihydroxycholecalciferol ($1,25(OH)_2D_3$ or calcitriol), which is produced by two hydroxylations of vitamin D, one in the liver (at position 25) and the other in the kidney (at position 1). These reactions occur whether vitamin D3 comes from the diet or from UV irradiation of 7-dehydrocholesterol in the skin. The most striking effect of calcitriol is its control of the expression of the gene encoding CaBP, causing the synthesis of the protein, thereby regulating the migration of calcium across intestinal cells. Calcitriol also has a "liponomic" action, increasing membrane permeability and activating the Ca-ATPase [9–11]. Calcitriol behaves like a hormone. Its renal production is regulated by parathyroid hormone (PTH), the secretion of which is, in turn, stimulated by a fall in plasma calcium concentration, which may itself stimulate calcitriol synthesis. The PTH-calcitriol system is also involved in bone resorption and increases the reabsorption of calcium by the renal tubule. This hormone system therefore controls all the calcium that enters the extracellular pool of exchangeable calcium and ensures that the plasma calcium concentration varies little from 100 mg/L.

The rate of saturable, physiologically regulated active absorption is negatively correlated with dietary calcium intake. Newborn babies lack this active process, and old animals (most studies have been done on rats) have calcitriol receptors, but they are less abundant than in younger animals and the renal 1-alpha-hydroxylase is less active; this is also the case in elderly people [12].

Supplementing the diet with vitamin D is not always allowed (it is forbidden in France), so most vitamin D comes from UV irradiation of the skin. However, the recommended daily dietary intake of vitamin D for adults is about 400 IU (10 micrograms).

Some of the membrane and cytosolic proteins involved in calcium transport are not vitamin D-dependent. One such protein is calmodulin, and others may be dietary proteins like alpha lactalbumin, which may act like calmodulin [9]. Apart from vitamin D deficiency, these are the only dietary means of affecting this highly regulated physiological route of calcium absorption.

Passive Diffusion. Passive absorption down an electrochemical gradient occurs via intercellular junctions or spaces. It involves the mass movement of water and major solutes such as sodium and glucose. It is not saturable and therefore increases with dietary intake, provided that the calcium in the intestines is in an absorbable form. It is independent of vitamin D and age [9–11].

All components of the diet that make calcium soluble or keep it in solution within the ileum should stimulate passive diffusion. Several molecules do this, particularly milk proteins like the phosphopeptides derived from casein [13,14] and amino acids like L-lysine and L-arginine, which form soluble chelates with calcium [10]. Lactose and other carbohydrates, which are gradually absorbed, also have an effect but the mechanism involved is still a matter of controversy. It is now generally agreed that lactose, at least in high doses, increases the passive absorption of calcium in the absence of vitamin D and, consequently, decreases intestinal CaBP concentration and active transport of calcium [15].

All molecules that increase the osmolarity of the liquid in the ileum are likely to stimulate the passive diffusion of calcium [15], whereas certain amino acids act on the intercellular space causing contraction of the cytoskeleton [11].

Other dietary factors make calcium irreversibly insoluble at near-neutral pH values, by converting it into forms such as phosphates, oxalates, phytates and soaps, which prevent passive absorption in the ileum. A variety of dietary factors have been shown to affect the passive diffusion of calcium, and this is a promising area of research aimed at producing the "extra" absorption that is generally desired.

Excretion, Retention and Balance of Absorbed Calcium

Most retained calcium is stored in the skeleton (99% of the body's calcium), depending on its needs. The main factors affecting the efficiency of calcium storage in bone are not dietary; they are physiological, related to growth, pregnancy and lactation, for example. The deposition and resorption of bone are regulated by several hormones (e.g., PTH, calcitonin, calcitriol and estrogens), the actions of which are outside the scope of this review. Excess absorbed calcium that cannot be stored in bone is excreted in urine, feces and sweat. The calcium balance in adult humans is zero, so all absorbed calcium is excreted by these routes, possibly after being incorporated into and then released from bone.

Almost all the calcium reabsorbed by the intestinal tract comes from secretions like the bile, and the endogenous calcium excreted in feces is the fraction that is not reabsorbed.

The urinary loss results from glomerular filtration (about 10g Ca per day) and tubular reabsorption, which retrieves over 98% of the filtered load [16]. Like intestinal absorption and bone exchange, the renal calcium flux is regulated by hormones, tubular reabsorption being particularly tightly regulated by PTH.

The amounts of calcium in human urine are much larger than those in the urine of other animals. Changes in the amount of calcium excreted in the urine may therefore have a major impact on calcium balance [17]. Certain dietary factors can influence the tubular reabsorption of calcium (see below), and these must be carefully noted when evaluating calcium bioavailability.

Fig. 1 shows the main pathways of calcium in adult humans. Human adults lose approximately 0.3% of their bone mass each year: this means that their calcium balance is negative and they lose about 10 mg of calcium each day. This loss of bone mass may be ten times greater in post-menopausal women.

The ultimate goal of all hormonal regulation of intestinal

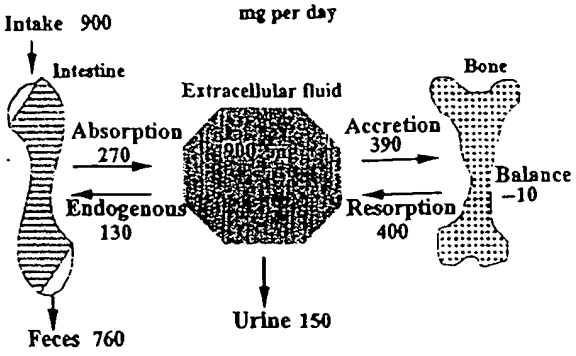

Fig. 1. The main pathways of calcium in adult humans.

absorption, bone resorption and renal tubular reabsorption of calcium is to keep the plasma calcium concentration constant, particularly the 50% of calcium in the ionic form. PTH and calcitriol are the most important hormones in calcium homeostasis. This complex control mechanism also regulates extracellular calcium, of which there are about 900 mg in the human body. Extracellular fluid (ECF) contains about 10^{-3} M Ca^{++}; the concentration of calcium ions in the cytosol is more than a 1000 times lower [16].

DIETARY FACTORS INFLUENCING CALCIUM BIOAVAILABILITY

This review covers only exogenous factors associated with the diet. Other endogenous factors like age, physiological condition and hormonal regulation have been discussed earlier [4–6,17] and are not discussed here. The main cause of changes in the rate of absorption and retention of calcium is clearly dietary intake, and there is an inverse relationship between intake and utilization. These changes have little to do with potential bioavailability, which is not controlled by hormones and does not reflect the absorptive capacity of the intestines or the retentive capacity of bone.

Dietary Factors Influencing Intestinal Absorption

Some components of the diet, such as the phytates found in bran and most cereals and seeds, oxalates in spinach, rhubarb, walnuts and sorrel, and tannins (tea), can form insoluble complexes with calcium, thereby reducing its absorbability. This only seems to affect calcium balance if the diet is unbalanced, high-fiber strict vegetarian diets lacking dairy products (calcium), for example. This must be taken into account when comparing dairy products with soybean-based products, which are generally phytate-rich. The apparently negative influence of fiber on calcium absorption is mainly due to the phytates that are frequently associated with dietary fiber. Other plant compounds, lightly methoxylated pectins for example, strongly inhibit the absorption of calcium and other minerals [18]. Fibers themselves (cellulose, hemicelluloses, lignins and non-cellulose polysaccharides) seem to have no direct effect on calcium absorbability. Some indigestible carbohydrates and hard-to-digest oligosaccharides have been shown to increase calcium absorption in the distal intestine by enhancing bacterial fermentation, thereby lowering the pH [19]. The effect of fibers and phytates has been examined in several reviews [e.g., 4,20]. A relative excess of phosphate has been thought to increase the fecal excretion of calcium. However, contrary to this widely held view, excess P does not reduce calcium absorption, at least if calcium intake is adequate. All Western-type meals have a Ca/P ratio well below 1, which favors the precipitation of calcium. This does not, however, prevent the normal absorption of calcium. Furthermore, the calcium in calcium phosphate is as well absorbed as the calcium in other inorganic salts, whether eaten with or without lactose [21].

Lipids, especially milk fats, are thought by some to form insoluble soaps with calcium, reducing its bioavailability. However, although this chemical reaction is possible, it does not, in practice, interfere with calcium absorption [4]. The dietary soaps are dissociated at the low pH of the stomach and cannot reform until they reach the ileum, which is beyond the main area of calcium absorption. Fecal soaps are formed from free long-chain saturated fatty acids and unabsorbed calcium. The saturated fatty acids in milk and cheese can displace calcium from phosphates in the ileum, forming less soluble soaps which are excreted, but this has no effect on the absorption of ingested calcium [22].

Other constituents of food, particularly components of milk, are thought to favor the intestinal absorption of calcium and to keep it in a soluble form until it reaches the distal intestine, where it can be absorbed by unsaturable routes that are independent of vitamin D. The best known are lactose, proteins and phosphopeptides.

Many *in vivo* and *in vitro* studies on proteins and phosphopeptides have demonstrated a positive effect of these molecules on calcium absorption. Phosphopeptides, derived from the enzymatic hydrolysis of caseins in particular, have been shown to sequester calcium and other cations, protecting them from potentially precipitating anions like phosphates in the intestine [13,14,23]. Phosphopeptides therefore help to keep calcium in solution until it reaches the distal intestine, thereby facilitating its absorption by passive diffusion.

Whey proteins, such as alpha lactalbumin and beta lactoglobulin, also bind calcium. Alpha lactalbumin binds calcium very tightly, making it a true binding protein, like calmodulin. However, despite the sometimes spectacular effects of these proteins and peptides on the solubility of calcium in the intestines *in vitro*, they have a much less dramatic effect on calcium absorption and retention *in vivo* [3].

The beneficial effect of lactose on the absorption of calcium and other cations has been more intensively studied than the effects of any other components of milk since it was demonstrated in rats by Bergheim in 1926 [24]. Interest increased

following the studies of French [25–27] and American [28–32] groups in the 1950s on the "lactose effect". The scientific debate on this issue is well described in the review by Miller [5], which is very well documented, but still incomplete.

It was first thought by the group of Fournier that lactose and other "structural" sugars acted directly on bone like precursors of bone proteins. This notion was replaced by theories of an intestinal action [7,28,29]. It is now clear that lactose, like other slowly absorbed sugars, must be at the site of absorption [28], that it prolongs the passive, vitamin D-independent absorption of calcium in the ileum [5,33] and that the effects of this action may be spectacular (doubling absorption) if a high dose of lactose (15% to 30% of the diet) is given.

Several theories have stressed the importance of keeping calcium soluble in the distal part of the intestines by forming soluble chelates [34] or by competition with inhibitors, such as phosphates. Fournier *et al.* [26] studied the effect of competition between lactose and phosphate on calcium absorption: lactose, like any other sugar that can be phosphorylated, accepts a phosphate group in a reaction catalyzed by alkaline phosphatase, thereby reducing the inhibition by phosphates within the lumen of the intestines. These authors therefore provided an explanation of why lactose in milk has little effect: the lactose and phosphate in milk have opposing effects.

It has been shown, however, that lactose does not act by increasing the concentration of soluble calcium in the lumen or by increasing the solubility of calcium phosphate *in vitro* [30]. There is also no cotransport of lactose and calcium [32]. The American group supported the idea that lactose acts on the intestinal mucosa to increase its permeability. All high osmolarity solutions double or triple the passive diffusion of calcium, probably by increasing the space of the intercellular junctions. This simple explanation may account for the effect of high doses of lactose [11]. Other studies [7,35] have shown that lactose and other sugars increase the absorption of calcium in the jejunum proportionally to their effects on water and sodium absorption.

The reduced bone resorption leads to the inhibition of bone turnover [27] in rats fed a lactose-enriched diet. This is caused by a large increase in intestinal calcium absorption [5]. However, the rat is not the appropriate model in which to study human bone remodeling (see below).

The effect of lactose has been clearly demonstrated in many experiments *in vitro* and in short- and long-term trials in rats, but its significance for human nutrition is much less clear [5,21]. Paradoxically, lactose, at least at physiological concentrations, does not seem to significantly affect the absorption of calcium from milk [36,37]. Only very high doses of lactose (50 g/day) have a net effect [7,38]. Calcium from yogurt, in which lactose is partially hydrolyzed, or from cheese, which contains no lactose, is absorbed as efficiently as that from milk [22,40,41,105].

Thus, lactose, at the concentrations normally found in milk, seems to have no significant effect on calcium absorption in healthy adults on a normal diet [5]. However, any effect of lactose on passive absorption may be masked by active transport, which is generally sufficient if the dietary intake of calcium is moderate and there is no lack of vitamin D. Lactose may be more important if calcium intake is high, especially in babies and the elderly, in whom solubility is a limiting factor and passive absorption is the predominant route [35]. Lactase deficiency does not prevent the calcium in milk from being well absorbed [36,41,42]. According to the excellent review by Scrimshaw and Murray [43] on lactose intolerance, which is prevalent in most of the world, with the notable exception of people originating from western and central Europe, even alactasic subjects can tolerate 250 g of milk per day and, thus, benefit from its calcium.

Meals have a major effect on the absorption of insoluble calcium supplements like calcium carbonate. Calcium carbonate is better absorbed when given as part of a meal than when it is given without food, particularly in fasting subjects. This has been clearly shown in humans [44] and in pigs [45] and is likely due to the calcium's being dissolved by the gastric juices and to slower gastric emptying.

Dietary Factors Influencing the Excretion of Calcium in Urine

Contrarily to the simultaneous intake of phosphorus, which can be confused with the meal effect (all common foods are rich in phosphorus), and certain constituents that raise the pH (bicarbonate, potassium salts), all the other dietary factors that have an effect at the kidney level increase the urinary loss of calcium generally by reducing tubular reabsorption [46].

Phosphorus may have a direct effect by increasing the reabsorption of calcium in the distal part of the nephron or an indirect effect by stimulating PTH secretion or by enhancing the uptake of absorbed calcium into bone [47]. The simultaneous absorption of calcium and phosphorus increases the uptake of calcium by bone, thereby decreasing its loss in urine [45].

Excess protein generally leads to an increase in the amount of calcium lost in the urine, which may be masked by the opposing effect of excess P (from dietary components rich in both protein and P). This is especially true for proteins with high contents of sulfur-containing amino acids (cysteine, methionine), the breakdown of which releases sulfur oxidized as sulfate, causing moderate acidosis and increasing the excretion of calcium in the urine [48,49]. Sulfate ions also bind calcium, preventing its tubular reabsorption [46,50–52] and even its incorporation into bone [53]. It is therefore not surprising that an excess of protein rich in sulfur amino acids or other sources of sulfate (certain mineral waters) causes more calcium to be lost in the urine than other foods, such as those eaten as part of a vegetarian diet or with bicarbonates [49,54,55].

Chronic metabolic acidosis due to excessive intakes of sulfate and chloride anions leads to higher losses of calcium in

the urine. The alkalosis resulting from ingestion of bicarbonate or potassium citrate has the opposite effect [55].

It has long been known that the renal clearance of calcium is linked to that of sodium. As almost all ingested sodium is excreted in the urine, this effect is particularly sensitive and several groups have developed equations describing it [46,56–60]. According to these equations, every extra two grams of dietary sodium increases urinary calcium excretion by an average of 30 to 40 milligrams.

Clearly, dietary factors affecting the amount of calcium lost in the urine have a major influence on calcium balance and may even be more important than those that influence the intestinal availability of calcium [17]. This is why the inevitable loss of calcium in the urine (accounting for a large part of the maintenance requirement) is greater for Western-type diets that are high in unfavorable factors such as animal protein, sulfates, sodium, coffee, tea and alcohol, than for other diets with lower levels of consumption of these factors.

METHODS FOR MEASURING CALCIUM BIOAVAILABILITY

In vitro Tests

Solubility in a slightly acidic medium is necessary, but not sufficient for bioavailability. The first step in the absorption of certain insoluble calcium supplements, given as tablets, is their disintegration and dissolution in the stomach. The solubility of $CaCO_3$ tablets is investigated using a kinetic test (USP) of dissolution in acetic acid (vinegar) in the US. Other primary tests of absorbability use dialysis, ultrafiltration and various membrane techniques, particularly the isolated intestinal loop, pieces of mucosa or cell layers (caco-2 cells). None of these methods takes into account the whole range of nutritional, physiological and ecological factors that influence absorption, and none provides results directly comparable with those obtained *in vivo* using whole animals.

Classical Balance Studies

This is the only method that provides true, absolute data on absorbability and bioavailability. It also gives mean values, although these are only valid for the period tested, and the test period must be at least one week after starting the diet (but several weeks are often needed). The balance method provides data for apparent (intake—fecal) absorption and net retention (intake—fecal—urinary) and the corresponding coefficients. Isotope dilution studies, using a stable or radioactive isotope of calcium, injected at the start of the evaluation, give the fecal loss of endogenous calcium and, hence, true absorption (intake—exogenous fecal).

Balance studies are slow, labor-intensive and expensive. The validity of the results obtained depends on how accurately the intake and output parameters are estimated. Even under the most rigorous experimental conditions (animals in metabolic cages) the inevitable small errors in assessment of intake (measured by excess) and fecal and urinary losses (measured by defect) always lead to an overestimation of the amounts retained. This overestimation may be very large when retention is low, as is always the case for adults.

Balance studies are essential for estimating the dietary needs of growing animals by the factorial method, but they are of little use for studies on human adults. Even under the most rigorous conditions (several days in a metabolic unit), the measurements are poorly reliable. Consequently, too much of the work published on human adults (normally in negative balance or equilibrium) has indicated that the individuals tested had a positive daily calcium balance as high as 200 to 300 mg calcium, which is most unlikely.

Fortunately, it is not necessary to carry out absolute balance studies or obtain absorption and retention coefficients for the comparison of several dietary sources of calcium. This can be achieved with values given relative to a reference source. This method only provides the bioavailability of calcium for an average diet over the test period for human subjects and cannot be used to compare two sources of calcium. Calcium sources can only be compared if the calcium is given as a single load, a test meal, giving the bioavailability of calcium at that time point only. One method used for human subjects [61,62] involves a preliminary intestinal lavage with isotonic solution followed by the test meal and, then, 12 hours later, a second intestinal lavage to collect the unabsorbed fecal residue. This rather drastic and unphysiological method has been used to show that there is little difference in the bioavailabilities of soluble and poorly soluble calcium salts [63].

Isotope Balance Methods

As in classical balance studies, all feces and urine must be collected over a period of several days, but the balance is calculated only on the tracer isotope in the source of calcium being studied. The intake is known accurately because it is a single dose of radioactive (^{45}Ca, ^{47}Ca) or stable (^{42}Ca, ^{44}Ca, ^{46}Ca or ^{48}Ca) isotope given in a test meal. The absorption and retention coefficients obtained are regarded as being representative of all the calcium in the labeled source.

Unlike the classical balance, the isotope test measures only the instantaneous bioavailability of a single dose taken as part of a meal. There is generally no period of adaptation, and variations over time are not taken into account, even though the coefficient of variation between meals and between days is probably over 10% [64]. The results obtained depend greatly on the experimental protocol, particularly the timing of the operations, such as whether the isotope is given to the fasting subject before, with or after the meal.

One of the main problems with assessments involving isotope tracers (see below) is the labeling technique itself. Ideally, an intrinsic marker should be used: for example, calcium in

milk can be labeled by giving the cow several injections of ^{45}Ca, whereas plant calcium can be labeled by adding the isotope to the fertilizer. Most labeling is extrinsic, however; this means that the food to be studied is mixed with the isotope, $^{45}CaCl_2$, for example. This assumes that there is a perfect exchange between the calcium in the foodstuff and the added isotope. Most dairy products seem to come rapidly to equilibrium [65], as do many other foodstuffs [66], but this is not true for certain plant products that contain insoluble calcium salts such as phytates and oxalates [67]. The bioavailability of calcium in these foods may therefore be considerably overestimated.

Urinary Excretion of an Oral Calcium Load

This is one of the methods most frequently used to compare sources of calcium in human studies. Unlike some animals (e.g., rats and pigs), which lose little or less calcium via the urine, humans excrete large amounts of calcium in urine. The increase in the amount of calcium lost after a calcium load is given to a fasting subject (about 500 mg Ca) may be thought of as reflecting the effectiveness of calcium absorption. However, the results reflect instant absorbability and also depend on several dietary factors that affect the loss of calcium in urine, by reducing it (phosphorus) or increasing it (sodium, high-sulfur protein, sulfate, certain carbohydrates).

This test is simple and fast. A urinary response can be obtained three to four hours after ingesting the test meal, and the urinary calcium data (relative to urinary creatinine) can be used to compare different sources of calcium [68–70]. Variations on this test use test meals labeled with stable isotopes.

Measuring Isotopes Labeled in the Blood, Urine or Bone

This may involve a single label for the rapid comparison (two to four hours after a single oral load) of labeled sources of calcium by measuring (sometimes with kinetic studies) radioactivity or stable isotope enrichment in the blood, urine or bone (used particularly for animals). An estimate of true relative absorption may also be obtained by measuring the area under the plasma isotope concentration curve. The direct measurement of the radioactivity taken up by a representative bone is possible using the ^{47}Ca isotope (a gamma ray emitter).

The most commonly used method at present is a double-label method in which a test meal labeled with one Ca isotope is ingested and a second Ca isotope is injected intravenously. The behavior of this second isotope reflects, in principle, 100% absorption. The isotope concentrations are measured later two to four hours in the blood, 24–36 hours in the urine). The ratio of the two isotopes (ingested and injected) is assumed to be equal to the fractional absorption (between 0 and 1) of the test calcium. Several sources of calcium can be compared rapidly and absolute true absorbability determined over several days without collecting feces. In addition, as these assays are relatively short in duration, they can be repeated on the same subjects after allowing for a "decontamination" interval.

This double radioisotope labeling technique has been widely used in animals and in humans [71]. A rapid method in which one radioisotope of calcium is injected, followed by a second injection of the same isotope 2 hours later has been devised by Chanard *et al.*, [72] and used routinely by Wynckel *et al.* [73]. Stable isotopes are now used in double-label studies in humans [74]. The validity of several variations of this method, differing in the type of blood sample or urine sample used and in the method of calculation, has recently been analyzed [75].

Several accurate mass spectrometry methods for measuring the enrichment of stable isotopes of calcium are now available [76]. The validity of bioavailability assessments based on these techniques depends, however, on several factors, including the quality of labeling of the test load, the representative nature of the samples and variations in the physiological and nutritional status of the experimental subjects.

Long-Term Evaluation of Bone Mineralization

Measuring bone parameters after prolonged treatment (several weeks for growing animals) is undoubtedly the most reliable way of estimating the long-term effects of qualitatively and quantitatively different calcium intakes. The mineral content, mineral density, breaking strength and morphometric parameters of a representative bone can be measured for experimental animals once they have been killed. The best methods currently available for measuring bone mass in several parts of the human skeleton are double X-ray absorptiometry (DEXA) or quantitative computed tomography (QCT) for lumbar vertebrae.

These bone criteria are generally sensitive enough for comparing sources of calcium, provided that the subjects are young, and reactive, with large calcium requirements and that they are assayed over a sufficiently long period. A single calcium intake concentration can be used for several sources, but it is better to use several intake concentrations for each source. This provides bone responses that vary with the intake. The slope-ratio of the curves for each source gives the bioavailability. This method gives very good results because it eliminates the large effect of small changes in calcium intake.

It is easier to interpret these data if the basal diet is low in calcium, because then almost all the calcium ingested comes from the test source, provided that all the other dietary factors affecting calcium absorption, such as proteins, phosphorus, phytates and sodium, remain the same.

Measuring of Biological Markers in the Blood or Urine

The concentration of PTH in the plasma falls when there is a small transient increase in plasma calcium concentration (or

in Ca^{++}) due to the intestinal absorption of an oral calcium load. This transient decrease is, however, proportional to the efficiency of absorption. This test is easy to perform in short-term comparative studies on human subjects, but it does not take into account further urinary loss of calcium, hence its retention by bone.

Some factors in the blood or urine vary with the degree of bone accretion or resorption. They can therefore be used in comparative tests to measure the effect of various amounts of absorbed calcium. The loss of hydroxyproline in urine is an indicator of bone resorption. It is now used in complement with or has been replaced by assays of more specific bone markers, collagen "cross-links", pyridinoline or, better still, deoxypyridinoline.

SELECTION OF ANIMAL MODELS

In vitro tests can be used to detect factors likely to alter the intestinal absorbability of calcium, but they are not really of use for quantifying bioavailability and cannot replace *in vivo* trials on animals. Experiments in humans are, of course, ultimately required, but it is still necessary, for many reasons, to carry out animal studies.

Selecting a Species

The main species used are rats, pigs, guinea pigs and primates. The dietary behavior of the animal must be taken into account, including the type of diet and frequency of meals, for example. Pigs are omnivores that eat rapidly two or three meals per day. This similarity to human behavior makes them an ideal model. Rats and guinea pigs eat grain and are continuous nibblers or gnawers without well defined meals.

The physiological characteristics of the rat also make it an unsuitable model. Its intestine presents high levels of phytase activity enabling it to hydrolyze phytates in food and to absorb calcium down as far as the large intestine. Neither pigs nor humans are able to do this, at least not to the same extent. The main problem with guinea pigs, rabbits and, to a lesser extent rats, is that they are coprophagous, a circumstance which makes interpretation of true absorption results complicated.

Rats are poorer animal models than pigs for studies on bone metabolism because their skeletons are continuously growing and never reach a bone remodeling stage paralleling that of human adults. In pigs, closure of epiphyseal cartilage occurs at the age of two to four years [77]. There is, however, no evidence that this difference, which may be important when studying factors affecting osteoporosis [78], has any effect on the absorptive capacity of the intestine.

Pigs and rats lose very little calcium in the urine, whereas humans and guinea pigs have very high urinary calcium levels. This factor limits the suitability of pigs for use in studies on the factors that may influence urinary calcium levels in humans. The lack of renal excretion of the excess absorbed calcium is probably offset in pigs by greater elimination via the endogenous fecal route.

Interest and Advantages of Animal Experiments

There is no doubt that it is preferable to carry out experiments on animals than on humans for ethical, material and financial reasons. Clearly it is much more feasible to work with young growing animals, whose calcium metabolism is very active, than to attempt such studies on children. Such experiments are important because the coefficients of calcium absorption and retention often depend more on the physiological condition of the subject than on the nature of the calcium ingested. Comparative studies on the bioavailability of several sources of calcium must therefore make use of subjects that are physiologically capable of retaining the ingested calcium.

Several technical manipulations are possible, such as the insertion of intestinal cannulae or catheters for repeated blood sampling. Radioisotopes are less expensive to use than stable isotopes, and they are also easier to measure accurately. Long-term trials involving extended periods in metabolic cages can be used to accurately measure ingested and excreted amounts; such trials are far from easy in humans. It is also possible to take organ samples from animals killed at a specific stage, which is of particular value for representative bone samples for a range of chemical, biological, morphometric and histological tests and for mechanical tests of breaking strength.

The power of the statistical tests that can be used is much greater with animal models. It is easy to set up very uniform groups with most of the animal species used (except, perhaps, primates), with very little variation between individuals and parameters like breed, strain, gender, age, weight, physiological state and dietary history all the same. Dietary components may be altered as required and the amounts consumed and excreted are accurately known.

It is thus possible to detect small but statistically significant differences between groups of ten individuals for animals, whereas dozens or even hundreds of individuals per group would be required if the experiments were done on humans. For example, we know that the average urinary loss of calcium in a human adult is 150±50 mg Ca/day (coefficient of variation=30%). Therefore, an increase of 15 mg per day in response to a dietary factor (e.g., sulfate) can only be statistically significant if the trial includes at least 100 subjects per group in a long term cross-over trial or many more subjects per group if it is a short-term trial with two groups. In contrast, this type of small effect is readily demonstrated in animals and has a very considerable long-term physiological consequence. Among other examples, the recent experiment done by Couzy *et al.* [74] shows the limits of human experiments. They concluded that sulfates in mineral water had no effect on urinary calcium loss, relative to milk, because the observed 14% increase was at the limit of statistical significance. In fact, because of the large

variation between individuals that is inevitable in this type of study, such a difference between two groups containing only nine adult subjects cannot be significant. However, the increases in urinary sulfate (+35%) and magnesium (+18%) were significant at the 5% level.

Only animal experiments can be used to show the statistical significance of small changes, and their demonstration in animals indicates that they may also exist in humans.

COMPARATIVE BIOAVAILABILITY OF CALCIUM IN FOODS: REVIEW

Comparison of Sources of Calcium

Human Studies. Many trials have been carried out over the past 15 years to compare calcium in milk with several other sources of calcium, such as salts, mineral waters and plant products. Almost 20 of the studies on bioavailability were carried out on men or women, using a variety of methods (true or apparent absorption, urinary calcium). None of the studies showed that the calcium in milk was more efficiently used than any calcium salt. Carbonate, gluconolactate, citromalate (CCM), chloride, lactate, acetate and citrate were tested [40,44,62,79–88]. The calcium from mineral water, bicarbonate or sulfate, was not found to be any better for absorption [73,74,89,90]. The findings were similar for several milk derivatives (yogurts, cheeses, chocolate milk, acidified milk) [40,70,90,91]. The calcium in milk and dairy products is much better absorbed than the calcium in spinach or watercress, as these plants have high oxalate contents [64,84,92–96]. Studies in humans, comparing the absorption of calcium from milk with that of CCM, suggest that calcium availability from CCM is higher [84,87], even than that from calcium carbonate [33,44,84,87]. A study carried out on women showed that the fractional absorption of calcium from cabbage was better than that of calcium from milk [98].

Studies on Rats and Pigs. There have been about 15 studies performed on rats over the past 15 years. They show a similar pattern, but many also include measurements of bone retention of labeled calcium [27,39,65,99,101–103] and tested a wider spectrum of minerals and milk products as sources of calcium that would be possible in studies on humans. Thus, studies in rats show that the calcium in whey is as efficiently absorbed and utilized for bone mineralization as that bound to casein [104,105] and that there is little difference between dairy products in general (milk, acidified milk, yogurt, skim milk, cream cheese, hard cheeses) [27,65,100,106]. Two studies in rats found differences between the "calcium value" of yogurt and milk [102,107], but their findings are contradictory. Studies in humans have shown that calcium absorption from these two sources is similar [40,41,91].

Long term studies in growing pigs [108,109] or ovariectomized mini-pigs [110] have provided no evidence that calcium is better absorbed from milk and milk products (casein phosphopeptides, skim milk or yogurt) than from mineral salts (CCM, $CaCO_3$). However, bone mineralization (evaluated by breaking strength) is better in animals fed yogurt as a calcium source than in those that obtain their calcium mainly from minerals ($CaHPO_4 + CaCO_3$) [109].

As in humans, most trials in rats have found no difference between the use of Ca from yogurt and that from other milk or mineral sources [27,40,41,65,91,100]. However, adding yogurt to the diet improves the fractional absorption of calcium [111]. Calcium in cheese is as efficiently used as the calcium in milk or carbonate [40,65,70,91,106]. A study on growing rats by Dupuis *et al.* [27] found that calcium is initially better retained from milk products than from calcium carbonate, but that this difference is later lost. Calcium from plants (apart from cabbage and some other crucifers), particularly that from cereals, is generally less well absorbed than the calcium from milk [112–114]. Phytates (present in large amounts in wheat bran and in soybean-based products) reduce the absorption of calcium from calcium carbonate [115] and from milk [116]. A study on rats using goat milk products found that the calcium from goat's cheese is less well retained than that from milk [65]. Only one study in rats found that increasing the dietary calcium intake with calcium sulfate leads to an increase, in four weeks, in bone mineral content. The same calcium intake from milk provides similar (ash as % dry matter) or higher levels of mineralization (Ca as a % of bone dry matter) than that provided by calcium sulfate [113]. The bioavailability of calcium from milk was estimated to be 113% that of calcium from calcium sulfate in this study.

To summarize. The mean apparent calcium absorption (% intake) from all collected data concerning calcium salts from human studies varied from 23% to 37%, excluding phosphates, because of the too large range of variation and the paucity of data. The following averages have been calculated from 3 to 8 references (citrate, citromalate, chloride, lactate, gluconate or a mixture of lactate and gluconate) to 12 to 14 references (carbonate, milk): carbonate, from 26.4 (fasting) to 29 (meal), citromalate from 32 (fasting) to 37 (meal), citrate 23.5 (fasting), lactate+gluconate 24.5 (fasting), chloride 30.6 (fasting), milk 32.4, cheese 32.8, mineral waters 32.3, oxalate-rich products (calcium oxalate, spinach, watercress) 13.2. These values are to be considered with care because they result from trials that compare different diets, ages and many other parameters. Furthermore, as suggested above, some calcium sources have been well investigated and some not.

Calcium Absorption *versus* Bone Retention

Intestinal absorption does not necessarily reflect the bioavailability to the whole organism because calcium must be retained and used in bone formation and mineralization. Phosphorus must also be present for the production of hydroxyapatite (a complex tricalcium phosphate). The dissociation of

calcium intake from that of phosphorus (if, for example, the calcium source is not ingested with the meal and/or this source contains no P), may restrict bone mineralization. This has been known for some time and was recently confirmed in growing pigs, which are extremely sensitive to dietary mineral supplies [45,117].

Three sources of calcium, calcium carbonate, CCM and milk, have been extensively studied. They all ensure the efficient absorption of calcium and also, over a long term (one to four years), that calcium is retained and used for bone mineralization. This was reported by Prince *et al.* [118] in a study on menopausal women, in whom calcium supplements, given as $CaCO_3$ tablets or as milk, reduce the bone loss measured over a two year period. Similarly, Smith *et al.* [119] studied 169 women aged from 35 to 65 years who were given calcium carbonate supplements (or a placebo) for four years. The calcium carbonate supplements reduced bone loss around menopause (bone mineralization study) at 12 sites. A group of adolescents was given a calcium supplement (one g/day, from 900 mL milk or calcium carbonate tablets), and it was found that bone density, determined 10, 18 and 24 months later, was higher in those given calcium than in those given the placebo and that the milk and carbonate sources were equally effective [120]. Recently [121] it was reported that calcium-enriched foods significantly increased bone mass accrual in prepubertal girls, with a preferential effect in the appendicular skeleton and greater benefit at lower spontaneous calcium intake. Lastly, postmenopausal bone loss was reduced at the main sites of spongy bone (but not of cortical bone) by supplementing calcium intake with calcium carbonate or CCM in a two year study by Dawson-Hughes *et al.* [122]. CCM was found to be the most effective. Other salts have been used in long-term studies (tricalcium phosphate, glucono-lactate plus calcium carbonate) and shown to reduce bone loss or the incidence of hip fracture [123,124].

Such longitudinal clinical studies have yet to be done using mineral water as the source of calcium. Hence, there is, as yet, no evidence showing that calcium from mineral water is similarly effective. While several human studies indicate that calcium from these sources is as well absorbed as that from milk or calcium carbonate, the effect of prolonged mineral water consumption on bone mineralization is not yet clear. Only one study, that of Cepollaro *et al.*, [125] reported a positive effect of consuming a high-calcium bicarbonate water on the bone density of 45 menopausal women, after 13 months of this form of supplementation. The control group (who drank a low-calcium water) was given no calcium supplement (calcium intake: supplemented, 1500 mg/day; non-supplemented, 949 mg/day). Apart from this trial, there have only been very short-term studies (a few days) for high-calcium mineral waters, and such studies are too short to test for any bone effect [74]. Careful interpretation is therefore required: the efficient absorption of calcium from these high-sulfate, high-bicarbonate waters, similar to that of calcium from milk or carbonate, does not necessarily show that this calcium is as well retained by bone. A recent preliminary report [126] showed that giving a calcium supplement in the form of calcium-rich water to postmenopausal women for two months reduced bone resorption (determined by the excretion of markers of bone resorption), but that the effect was much less marked with high-sulfate water than with high-bicarbonate water. It is well known that the urinary loss of calcium is lower with alkalogenic diets, rich in vegetables and fruits or bicarbonates [54,127]. The problem of urinary loss of calcium with these calcium sulfate sources remains to be determined over longer periods.

Our recent studies in growing pigs have shown greater bone mineralization (measured as ash, density and breaking strength of various bones) in pigs fed a "milk" diet (70% of the calcium intake as powdered skim milk) than in pigs fed a "sulfate" diet (50% of total Ca intake as $CaSO_4$ and 33% as $CaCO_3$) or a "carbonate" diet (80% of intake). The sulfate and carbonate diets gave similar levels of mineralization. All the diets had the same energy, protein and calcium contents (Pointillart and Guéguen, unpublished results).

Casein Phosphopeptides, Proteins and Calcium Availability

The positive effects of milk casein phosphopeptides (CPP) on the absorption of calcium have been shown mainly with *in vitro* studies of calcium transfer (ligated intestinal loops or everted sacs) in rats [128–133] and in a few *in vivo* studies in rats in which calcium absorption and bone retention were measured [134–137]. The CPP were compared to soybean protein extracts, egg white [135], wheat gluten or gelatin [129] or fibrin [131]. A study on isolated chicken intestinal loops also showed that CPP increased calcium transfer [14]. A diet in which 50% of calcium and about 33% of P are provided by CPP has no effect on calcium absorption or bone retention in pigs [108]. Feeding of casein, a potential substrate to the release of CPP, to growing miniature pigs improved femur mineralization as compared to whey protein. This observation was true when vitamin D deficient diets were given but not when adequate vitamin D supply was provided [137].

Studies on unweaned babies show that those fed soybean-based formula have 25% less bone mineralization (from densitometry measurements) than those fed milk-based formula [138,139]. Conversely, *in vivo* studies on ovariectomized rats showed that these animals lost less bone if fed a diet containing soybean protein extract than if fed a milk-based diet [140, 141]. The authors interpreted this as being due to the phytoestrogens in soybean. It is difficult to extrapolate these results to humans, given that Tsuchita *et al.* [142] clearly showed less bone loss following ovariectomy in rats fed CPP than in rats given Ca and P as pure minerals.

Partridge [143] showed greater calcium absorption in very young pigs fed milk than in those fed an isocalcium diet containing soybean meal. Similar results were obtained in pigs

by Matsui *et al.* [144]. The opposite pattern is later observed (soyabean>milk) in pigs aged four months, and there is no difference in older animals (soya=milk).

The positive estrogen-mimetic effect on bone has only been observed in ovariectomized rats, and soybean products have a high phytate content which may reduce calcium absorption, as has been clearly demonstrated, including in women [116,145]. Lastly, several *in vivo* studies have shown that calcium in diets with various soyabean and CPP contents is similarly absorbed [rat: 104,146,147; pig:108]. A clinical study on unweaned babies up to six months old compared bone density at various stages of development, and found no difference between mother's milk and formulas based on soybean or on cow's milk [148]. In contrast, the amount of animal protein consumed by women was found to be strongly correlated with the incidence of hip fracture in a retrospective epidemiological study carried out by Abelow *et al.* [149].

It has been shown in many studies that the greater the amount of dietary protein, the higher the urinary calcium level, regardless of whether the protein is casein or soybean protein [in rats: 147; in man: reviewed by Abelow *et al.*, 149]. Thus, high levels of protein consumption lead to a negative calcium balance. Reducing the milk protein content of the diet reduces urinary calcium loss in man [150]. Calcium supplementation in the form of milk increases the amount of sulfate in urine because milk has a high content of sulfur-containing amino acids [80], and some studies have implicated these amino acids in the hypercalciuria and negative calcium balance associated with diets containing too much animal protein [51,55,147,151–153]. A horizontal study carried out in China on women who had consumed a variety of diets (with and without animal protein, plus or minus milk) indicated a greater correlation between urinary calcium and the consumption of animal proteins not derived from milk [154]. However, things are not that simple. A study performed by Allen *et al.* [155] on humans with controlled diets and for whom the dietary protein was tripled from 12 g N/day to 36 g N/day by adding soya extract clearly showed an increased urinary calcium loss (1.5-fold) which changed the calcium balance from −37 mg/day for 12 g N/day to −137 mg/day on 36 g N/day, despite high calcium intakes (1400 mg/day) and the similar absorptions. This problem of the effect of excess protein on bone has been recently discussed [55].

In Conclusion. While the proteins in milk or milk products may have beneficial effects on bone mineralization, this is not always so. In contrast, the positive effects of soya on calcium retention have only been demonstrated in one rather special system, ovariectomized rats, while it has been clearly shown that the phytates in soya can reduce calcium absorption in humans. Both milk and CPP have a favorable effect on calcium absorption. The high phosphorus content of milk may offset the hypercalciuria induced by protein [156], although the intakes of both calcium and phosphorus provided by the milk help promote bone mineralization.

The hypercalciuric effects of high-protein diets, particularly those containing animal proteins, are well known. However, human studies on the nature of these proteins, plant/animal, milk/non-milk proteins, and their long term influence on calcium balance or bone metabolism are still necessary before we can come to any conclusion about whether plant proteins are advantageous or not. Thus, particularly strict vegetarian diets that contain no milk products may present risks to bone mineralization [157] because they do not provide an adequate calcium intake, without recourse to supplements provided by mineral calcium tablets [114].

Lactose, Lactase and Calcium Bioavailability

In Rats. Lactose is reputed to stimulate calcium absorption and most of the experimental evidence for this has been obtained in rats [21,25,26,29,39,158]. These *in vivo* studies provide direct evidence that it acts on the intestines and on bone. There is also indirect, *in vitro*, evidence obtained from studies on isolated intestinal loops [31,159] in which lactose was compared to another sugar or the absence of lactose [159]. Other studies on isolated gut loops *in situ* have, however, shown that 30% lactose can reduce the absorption of calcium chloride [15]. There is also other indirect experimental evidence. For example, *in vivo* studies have compared the calcium absorbed from normal milk and from milk in which the lactose had been hydrolyzed [39]. Others have shown that lactose, unlike sucrose, reduces the effects of a lack of vitamin D on bone [5]. Lastly, adding lactose to cheddar cheese was found to give better calcium absorption than with cheese alone [106].

In Humans. The effect of lactose is perhaps less clear cut in man because it is complicated by the problem of lactose intolerance and thus of a lactase deficiency [see review by Scrimshaw and Murray: 43]. Griessen *et al.* [36] found that lactose increased the fractional absorption of calcium in lactase deficient (LD) patients, but most studies have shown a reverse effect [38,160–162] or no effect [37]. A group of five studies showed that the presence of lactose, or its addition, stimulated calcium absorption in lactose-tolerant subjects [35,38,161,163,164], but three other studies demonstrated no effect [37,165,166].

There is no real proof that hypolactasic patients absorb calcium less well than others. At least one study [37] found that the absorption of calcium from a standard diet by lactase-deficient patients was better than that of lactose-tolerant patients, another found that it was poorer [160], while still others have reported that the basal absorptions were similar [36,38,41,111], even when there was milk or yogurt in the diet [41]. Yogurt can increase calcium absorption in both LD and non-LD subjects [111] compared to a $CaCl_2$ solution.

Normal mother's milk results in better absorption of calcium by unweaned babies than when the lactose is removed, and adding lactase to mother's milk can increase calcium absorption [163]. Lactose seems to have an even greater effect

on calcium absorption when absorption is basically poor [35,164].

A recent clinical study [167] on children about 10 years old found no difference between the bone densities of lactose-intolerant and paired (height and weight) controls, but they did find that there was a strong correlation ($r=0.9$) between bone density and calcium intake in the lactose-intolerant children. Several epidemiological studies have also shown that lactose-intolerant subjects consume less calcium (from dairy products), which may predispose them to osteoporosis [168,169]. This link is not always found, as indicated by the lack of a difference between the bone densities of female twins, one of whom was hypolactasic and the other lactose tolerant (in response to an oral load) [170]. The answer may lie in the total amount of Ca consumed, i.e. from dairy and non-dairy foods.

In conclusion. Several studies have shown that lactose has a positive effect on calcium utilization, but there is some uncertainty, at least in LD people in whom lactose can reduce absorption. It is possible that this effect is only temporary, as suggested by certain studies on the changes in calcium absorption after a meal [38]. The literature contains many contradictions concerning the reduced absorption in LD subjects. It is quite probable that the non-consumption of dairy products by these subjects tends to reduce their calcium intake, but that could be offset by more efficient absorption, provided that they still have the capacity to adapt; it is far from clear that the elderly have such a capacity.

Influence of Dairy Product Consumption on Bone Density

All of the 14 clinical or epidemiological studies published over the past decade [118,171–181], except one [182], have shown that the consumption of dairy products in childhood and adolescence has a positive effect on bone mineralization later in life, as assessed by bone density measured at several sites in adults. They therefore confirm the classic findings of Matkovic *et al.* [183]. This effect on subsequent bone density is reduced or lost when milk is consumed between the ages of 20 and 30 [172,173,179]. Several studies have found that a dairy product supplement increased bone density in adolescents [174,177] or reduced bone loss in post-menopausal women [118]. Lastly, osteoporotic women were found to have consumed less dairy product than healthy controls when they were children and adolescents [172,175]. A recent review [184] done on children found that consumption of extra calcium increased their bone density 1% to 5% or even 10% when the source of calcium was dairy products. This was recently confirmed with a double-bind, placebo-controlled trial in prepubertal girls [121]. The question remains on whether such effects persist after six to 36 months of intervention.

Only one study, that by Prince *et al.* [118], has compared the effects of calcium supplements, given as 1-g day mineral tablets or dairy products, for two years on women at least 10 years after menopause. Both types of supplements have similar effects: they reduce bone loss from several sites in the hip, but not from the lumbar vertebrae. The findings of a number of recent meta-analyses of data from horizontal studies looking for a link between calcium intake and bone loss have arrived at contradictory conclusions. However, prospective studies on the effects of calcium supplements have generally shown that it has a positive impact on bone loss [185,186]. According to Nordin [187], some contradictory conclusions could be due to errors in the dietary data. Nordin analyzed 19 trials, three using dairy products. This analysis clearly showed that calcium supplementation reduced bone loss from 1.26% per year in controls to 0.12% in those receiving calcium supplements ($p<0.005$). These are data for the bone densities of 1300 postmenopausal women, measured at 11 bone sites, including cortical and/or trabecular bone. The difference between the annual percent bone loss between supplemented and unsupplemented women varied from +0.28 (spine) to +4.1 (femoral diaphysis). Lastly, Lyritis *et al.* [188] found a correlation between the consumption of dairy products by young adult humans and their bone density.

We can therefore say that a greater calcium intake, particularly of milk products, during the period of peak bone formation has a positive effect on bone density of adults and undoubtedly reduces the risk of osteoporosis. But only intervention studies have shown that calcium supplementation has a beneficial effect on bone loss, while the results of horizontal epidemiological studies are more controversial.

PECULIARITIES AND ADVANTAGES OF THE CALCIUM IN MILK AND DAIRY PRODUCTS

It is well worth remembering that milk and milk products are by far the main source of calcium in our diet [1]. Cow's milk contains an average of 1.20 g calcium per liter, 20% of which is bound to casein as an insoluble organic colloid and the remaining 80% in mineral form (45% in the tricalcium phosphate of the phospho-caseinate, which is also insoluble and colloidal, and 35% soluble, including 12% as ionized calcium) [189]. The organic or mineral calcium bound to casein is readily released during digestion, and there is general agreement that its potential bioavailability is high. Most solubility studies use milk calcium as a reference standard. The calcium in spinach, which is present as an insoluble oxalate, is taken as the extreme example of poor bioavailability. However, except for newborns fed on mother's milk (calves drinking cow's milk) which can absorb almost all the ingested calcium, the percent of milk calcium absorbed seldom exceeds 40% under normal dietary conditions.

The calcium in cheeses is readily available, despite the fact that cheese often contains large amounts of saturated long chain

fatty acids and no lactose [22]. Tests on rats fed cheddar cheese labeled with ^{47}Ca showed that the calcium was as well absorbed as was that from milk and that absorption was not influenced by the maturation time [106].

There is therefore no difference in the availability of calcium from milk and most of the best mineral or organic sources of calcium which are often used as medicines or dietary supplements and whose coefficient of absorption is about 30% to 40%. Only a few organic forms, like citrate-malate, can provide slightly better calcium availability [2].

Nevertheless, the calcium in milk differs in several interesting features from the calcium in other foodstuffs or supplements. These can be important when it is necessary to ensure high absorption of calcium under unfavorable physiological conditions [35]. Because it is bound to peptides and proteins, milk calcium is more likely to remain in solution when the pH is unfavorable, such as in achlorhydria. Milk calcium may be absorbed in the absence of vitamin D, under the influence of lactose in the distal small intestine via the paracellular route. Thus milk can provide calcium with "ensured absorbability" which is generally insensitive to external factors, except for inhibitors, such as oxalic acid. Dairy products do not contain anything likely to inhibit the intestinal absorption of calcium, like phytates, oxalates, uronic acids or the polyphenols of certain plant foods. The hypercalciuric effect of sulfates from milk proteins is offset by the hypocalciuric effect of phosphorus [156]. The endogenous sulfates produced by the breakdown of sulfur-containing amino acids produces a SO_4/Ca ratio of 0.6, while this ratio is 2.6 in some high-sulfate, high-calcium mineral waters.

Lastly, it should be remembered that milk and dairy products are not only excellent sources of calcium, but also provide an almost complete diet whose consumption provides a "meal effect" [17]. This fosters the absorption of calcium and provides a simultaneous intake of phosphorus that is essential for bone deposition. These advantages cannot be provided by any other source of calcium, such as calcium supplements or Ca-rich waters.

As milk provides calcium with "protected absorbability," "prolonged absorption" and "extended bone deposition," milk is the most suitable dietary constituent that meets the high calcium intake required by postmenopausal women and the elderly. This is especially important because, according to some workers [176], and for still unknown reasons, the inhibition of bone remodeling that generally occurs in response to a high calcium intake is less marked when calcium is supplied by milk products. Further studies are now needed to identify a possible specific effect of milk products on bone, although this beneficial effect could be simply due to different rates of calcium absorption, with slower gastric emptying and a prolonged passive diffusion that ensures an extended supply of calcium to the bone.

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TRANSMITTAL FORM

Application Serial Number	10/067,527
Filing Date	February 4, 2002
First Named Inventor	Takada
Group Art Unit	1654
Examiner Name	Gupta, Anish
Attorney Docket No.	FJN-058C1
Confirmation No.	8309

ENCLOSURES *(check all that apply)*

- ☒ Fee Transmittal Form (1 pg.)
  - ☒ Check Attached ($1130.00)
  - ☐ Copy of Fee Transmittal Form
- ☒ Amendment/Response (9 pgs.)
  - ☐ Preliminary
  - ☐ After Final
  - ☐ Affidavits/declaration(s)
  - ☐ Letter to Official Draftsperson including Drawings [Total Sheets _____]
- ☒ Petition for Extension of Three Month Time (1 pg.)
- ☒ Supplemental Information Disclosure Statement (2 pgs.)
  - ☒ Supplemental Form PTO-1449 (1 pg.)
  - ☒ Copy of IDS Citation (CL-18 pgs.)
- ☐ Certified Copy of Priority Document(s)
- ☐ Sequence Listing submission
  - ☐ Paper Copy/CD
  - ☐ Computer Readable Copy
  - ☐ Statement verifying identity of above
- ☐ Copy of Notice to File Missing Parts of Application (PTO-1553)
- ☐ Formal Drawing(s)
- ☐ Request For Continued Examination (RCE) Transmittal
- ☐ Power of Attorney (Revocation of Prior Powers)
- ☐ Terminal Disclaimer
- ☐ Executed Declaration and Power of Attorney for Utility or Design Patent Application
- ☐ Small Entity Statement
- ☐ CD(s) for large table or computer program
- ☐ Amendment After Allowance
- ☐ Request for Certificate of Correction
  - ☐ Certificate of Correction (in duplicate)
- ☐ Notice of Appeal to Board of Patent Appeals and Interferences
- ☐ Appeal Brief (in triplicate)
- ☐ Status Inquiry
- ☒ Return Receipt Postcard
- ☐ Certificate of First Class Mailing under 37 C.F.R. 1.8
- ☐ Certificate of Facsimile Transmission under 37 C.F.R. 1.8
- ☒ Additional Enclosure *(please identify below)*
  - ☒ Exhibit A – "The Bioavailability of Dietary Calcium;" *Journal of American College of Nutrition*, Vol. 19(2), 119S-136S (2000)(18 pgs.)

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FEE TRANSMITTAL
FY 2004

Complete if Known	
Application Serial Number	10/067,527
Filing Date	February 4, 2002
First Named Inventor	Takada
Group Art Unit	1634
Examiner Name	Gupta, Anish
Attorney Docket No.	FJN-058C1

METHOD OF PAYMENT

1. ☒ Payment Enclosed:
   ☒ Check ☐ Money Order ☐ Other

2. ☒ The Commissioner is hereby authorized to credit or charge any fee indicated below for this submission to Deposit Account No. 20-0531.
   ☐ Required Fees (copy of this sheet enclosed).
   ☒ Additional fee required under 37 CFR 1.16 and 1.17.
   ☒ Overpayment Credit.

3. ☐ Applicant claims small entity status.

FEE CALCULATION

1. FILING FEE

Large Entity Fee ($)	Fee Description	Fee Paid
770	Utility filing fee	
340	Design filing fee	
160	Provisional filing fee	

	Number Filed	Number Extra	Rate	Amount
Total Claims		- 20 =	x $ 18.00 =	
Independent Claims		- 3 =	x $ 86.00 =	
☐ Multiple Dependent Claim(s), if any			$290.00 =	

TOTAL:
SMALL ENTITY DISCOUNT:
SUBTOTAL (1) ($) 0.00

2. AMENDMENT CLAIM FEES

	Claims Remaining After Amend.	Highest No. Previously Paid For	Present Extra	Rate	Fee Paid
Total	-	=		x $ 18.00 =	
Indep.	-	=		x $ 86.00 =	
☐ First Presentation of Multiple Dep. Claim				+ $290.00 =	

TOTAL: ($)
SMALL ENTITY DISCOUNT: ($)
SUBTOTAL (2) ($)0.00

FEE CALCULATION (continued)

3. ADDITIONAL FEES

Large Entity Fee ($)	Small Entity Fee ($)	Fee Description	Fee Paid
130	65	Surcharge - late filing fee or oath	
50	25	Surcharge - late provisional filing fee or cover sheet	
130	130	Non-English specification	
2,520	2,520	Request for ex parte reexamination	
110	55	Extension for reply within first month	
420	210	Extension for reply within second month	
950	475	Extension for reply within third month	950.00
1480	740	Extension for reply within fourth month	
2010	1005	Extension for reply within fifth month	
330	165	Notice of Appeal	
330	165	Filing a brief in support of an appeal	
290	145	Request for oral hearing	
130	130	Petitions to the Commissioner	
180	180	Submission of Information Disclosure Statement	180.00
770	385	Filing a submission after final rejection (37 CFR 1.129(a))	
770	385	For each additional invention to be examined (37 CFR 1.129(b))	
100	100	Certificate of Correction for applicant's error	
110	55	Submission of Terminal Disclaimer	
Other fee (Specify)			
Other fee (Specify)			

SUBTOTAL (3) ($) 1130.00

SUBTOTAL (1)	0.00
SUBTOTAL (2)	0.00
SUBTOTAL (3)	1130.00

TOTAL ($) 1130.00

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Respectfully submitted,

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